# Differential response of normal and malignant urothelial cells to CHK1 and ATM inhibitors

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**I hereby declare that no part of this thesis has been previously submitted for any degree or qualification at this, or any other University or Institute of learning.**

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# Summary

This study focuses on the apparently distinct roles of various checkpoint associated kinases including ATM, ATR, and CHK1 in bladder cancer compared with normal human urothelial (NHU) cells. The primary signalling cascade that responds to replication stress in bladder cancer cells (ATR-CHK1) is deficient in normal urothelial cells after treatment with a replication inhibitor and these cells do not depend upon CHK1 for protection from apoptosis during replication stress. This is very different from the situation in bladder cancer cells, where ATR-CHK1 plays a prominent role. Instead ATM signalling is more rapidly activated under these conditions. Intriguingly, an ATM inhibitor suppressed S-phase checkpoint activation after exposure to replication inhibitors and stopped entry of hTERT-NHU cells into S-phase with $G_1$ accumulation of cells suggesting activation of a $G_1$ checkpoint. Consistent with this, cells treated with the ATM inhibitor and thymidine showed increased levels of cyclin-dependent kinase inhibitor $p19^{INK4D}$, reduced levels of cyclin D1 and CDK4, and reduced phosphorylation of the retinoblastoma (RB) protein. In contrast, a bladder cancer cell line treated with the ATM inhibitor progressed more slowly through S-phase and showed a dramatic increase in apoptosis following co-treatment with ATM and replication inhibitors. Taken together,

these findings suggest that ATM and CHK1 signalling cascades play different roles in tumour and normal epithelial cells, confirming these as promising therapeutic targets.

# Abbreviations

**AT** Ataxia Telagiectasia
**ATM** Ataxia telangiectasia mutated
**ATR** Ataxia telangiectasia and Rad3 related
**BSA** Bovine Serum Albumin
**CDK** Cyclin Dependent Kinase
**cDNA** copy Deoxyribonucleic Acid
**CO2** Carbon Dioxide
**DMEM** Dulbecco's Modified Eagle Medium
**DMSO** Dimethylsulphoxide
**DNA** Deoxyribonucleic Acid
**DSB** Double Strand Break
**ECL** Enhanced Chemiluminescence
**EDTA** Disodium ethylene diamine tetra-acetate
**EtOH** Ethanol
**FACS** Fluorescence Activated Cell Sorting
**FCS** Foetal Calf Serum
**hTERT** Human Telomerase Reverse Transcriptase
**HU** Hydroxyurea
**KDa** KiloDaltons
**M** Molar
**mA** Milliamps
**mg** Milligram
**ml** Millilitre
**mM** Millimolar
**μg** Microgram
**μl** Microlitre
**μM** Micromolar
**min** minute
**mRNA** Messenger Ribonucleic Acid
**MTT** Thiazolyl Blue Tetrazolium Bromide
**MW** Molecular Weight
**nM** Nanomolar
**OD** Optical Density
**PAGE** Polyacrylamide Gel Electrophoresis
**PBS** Phosphate Buffered Saline
**PBST** Phosphate Buffered Saline+Tween20

**PCR** Polymerase Chain Reaction
**pH**
**PI** Propidium Iodide
**PMSF** Phenylmethylsulphonylfluoride
**RNA** Ribonucleic Acid
**RPMI**
**RT-PCR** Reverse Transcription Polymerase Chain Reaction
**SDS**
**Ser** Serine
**siRNA** Short Inhibitory RNA
**ssDNA** Single Strand DNA
**TdR** Thymidine
**Thr** Threonine
**TEMED**
**V** Volts
**WT** Wild Type

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# CHAPTER 1 INTRODUCTION

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# Chapter 1 Introduction

## 1.1 Cancer

Cancer is a large family of genome diseases and one of the most serious health threats facing the world today. So far more than 200 distinct types of cancer have been determined. In addition, cancer can develop in any organ from almost any type of cell in over 60 different body parts. In general, cancer can be classified into three categories by the cell types of cancer development and further classified according to tissue of origin. Most of human cancers (about 90%) are malignancies of epithelial cells which are considered as carcinomas. The tumours of supporting tissues such as bone, cartilage, fat, connective tissue, muscle are sarcomas. These types of cancer are much more rare than carcinomas. Furthermore, human cancers originating from cells of blood & lymphatic origin are called leukemias and lymphomas and make an about 8% of all malignancies. The causes of cancer are diverse and complex. In the beginning, there are six hallmarks of cancer have been identified: 1. self-sufficiency in growth signals, 2. insensitivity to anti-growth signals, 3. evading apoptosis, 4. limitless replicative potential, 5. tissue invasion and metastasis, 6. sustained angiogenesis (Hanahan and Weinberg, 2000).

- Self-sufficiency in growth signals: It is generally accepted that normal cells require mitogenic growth signals for active proliferation from external surrounding. There is no type of normal cell that can keep proliferating in the absence of these growth signals. However many of the oncogenes in cancers were found to act as growth signals for their own proliferation.
- Insensitivity to anti-growth signals: In contrast to growth signals, there are also multiple anti-proliferative signals and mechanisms which are able to operate to maintain cellular quiescence for preventing uncontrolled growth within normal cells. For instance, transforming growth factor-beta (TGF-beta) was reported to inhibit cell proliferation by inducing a $G_1$-phase cell cycle arrest (Hannon and Beach, 1994). It is not surprising that cancer cells have developed mechanisms to evade those kinds of growth control.
- The evasion of apoptosis: Apoptosis, programmed cell death, is a critical mechanism to eliminate damaged or mutated cells which can not be repaired. It also represents a major source of cell attrition to control the cell number in tissues to avoid unlimited cell proliferation. Hence malignant tumour cells must therefore develop a means to avoid both intracellular and extracellular apoptosis signals.

- Unlimited replicative potential: Cell proliferation is strictly limited in normal cells. For instance, normal human cells carry an intrinsic finite replication limitation that relies on this shortening of their chromosome telomeres after cell mitosis. However telomerase, the enzyme which is able to maintain the length of telomere is re-activated in cancer cells. Most tumour cells in culture are immortalized suggesting that this limit of replicative potential does not exist in cancer cells.
- Tissue invasion and metastasis: Most types of human cancer cells are able to migrate to adjacent or distant sites and invade surrounding tissues from primary tumour site during early or later development stage. Although the mechanisms and details remain incompletely understood, the affected proteins include cell–cell adhesion molecules (CAMs) and extracellular proteases. These newly formed metastases are the causes of 90% of cancer death.
- Sustained angiogenesis: Unlike vasculogenesis which is the *de novo* formation of blood vessels (Risau and Flamme, 1995), angiogenesis refers to the process of new formation of blood vessels from pre-existing ones. The oxygen and nutrients carried by the vasculature are essential for cell growth and survival in both normal and malignant cells. Thus, in

order to progress to a larger size, angiogenesis is necessary for tumour cells.

Recently, these hallmarks of cancer were underlined as genome instability which generates the genetic diversity and further advances cancer development. For instance, mutated and constitutively activated growth factor receptors activate additional downstream signalling transduction such as Raf-MAPK pathway which participates in cell proliferation and invasion (Davies and Samuels, 2010). Null mutation occurring on tumour suppressor genes including Rb and TP53 results in tumour cells evade growth suppressors (Ghebranious and Donehower, 1998; Lipinski and Jacks, 1999). Furthermore, TP53 induces apoptosis by up-regulating expression of the pro-apoptotic triggering proteins such as Bax and Bak in response to DNA damage (Adams and Cory, 2007), thus loss of functional TP53 enable tumour cells to resistant cell death from stress.

In the past decade, two enabling characteristics such as “genome instability” including random mutations and chromosomal rearrangements and “tumour-promoting inflammation” have also been identified as cancer

hallmarks which contribute to tumour progression and development.

“Deregulating cellular energetic” and “avoiding immune destruction” are another two newly identified hallmarks of cancer, which involve in cellular metabolism or re-programme to neoplastic proliferation and to evade immunological destruction particularly by T and B lymphocytes, macrophages, and natural killer cells (Hanahan and Weinberg, 2011). So far, there are currently 10 typical hallmarks of cancer have been identified.

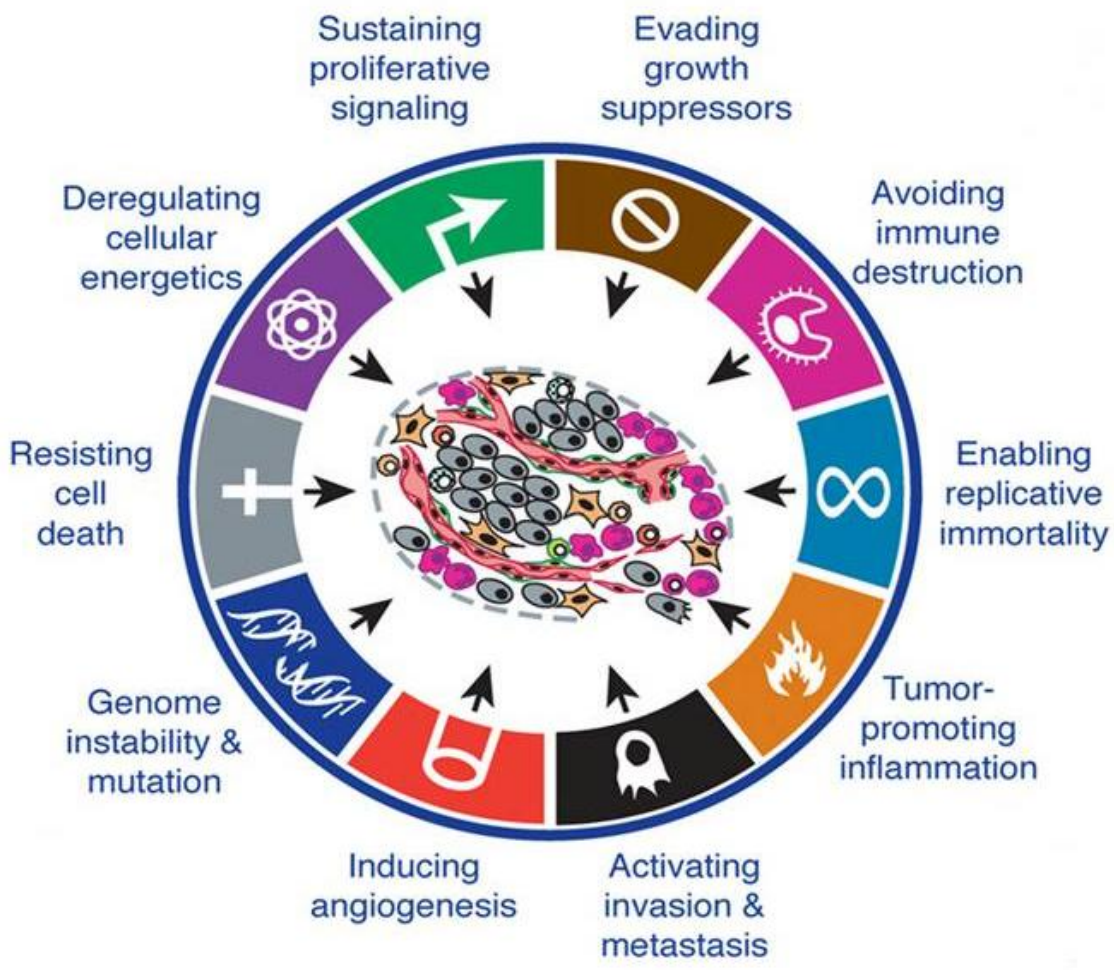


**FIGURE1. The hallmarks of cancer (Hanahan and Weinberg, 2011)**

Additionally, whereas the molecular basis of genomic instability in sporadic cancers is still unclear, mutations in DNA repair genes have been considered to result in genomic instability and further leading to cancer development in hereditary cancers. In hereditary cancers genomic instability is considered as the initial event, which further contributes all the other hallmarks. However, deregulation of growth signalling may be the initiating event in sporadic cancers. Deregulation of growth-regulating genes leads to DNA damage and replication tress and then causes genomic instability. The mutation occurring on tumour suppressor genes such as TP53, ATM and CDKN2A not only contributes genomic instability but also demonstrated to induce DNA damage (Negrini et al., 2010).

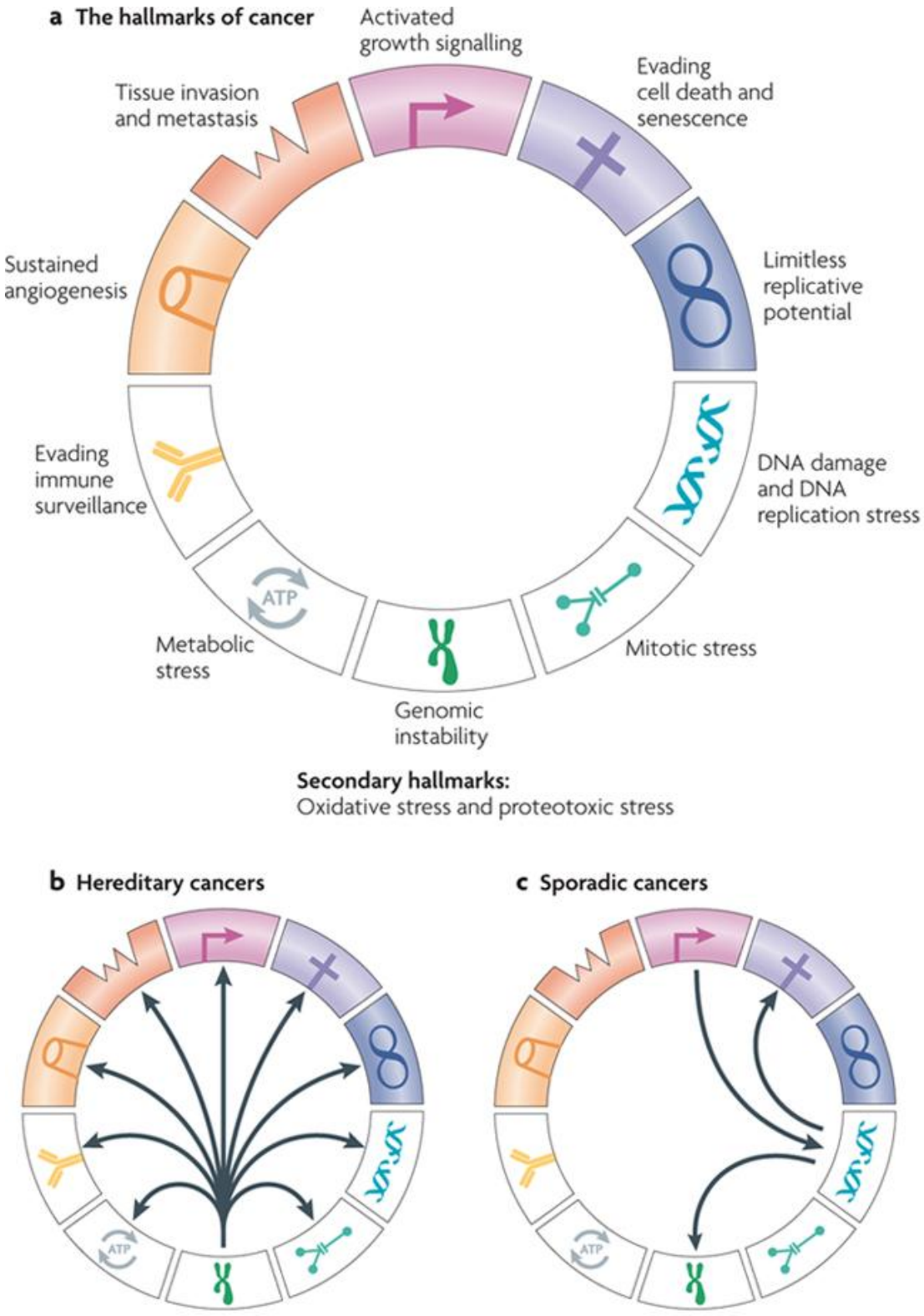


**FIGURE1.1 Genomic instability in hereditary and sporadic cancer (Negrini et al., 2010)**

### 1.1.1 Cancer Statistics

It has been reported form a statistical study of cancer research UK that malignant tumours are the leading cause of death around the world. More than 8.2 million people died because of cancer worldwide in 2012. Approximately 331,000 new cancer cases are projected to occur, resulting in over 161,000 deaths in the United Kingdom in the same year. Seriously, it is estimated that more than one in three people in the UK will develop some form of cancer during their lifetime. The incidence rates for all cancers combined have largely increased by 23% in males and 43% in females since mid-1970s in the UK. In other words, cancer is one of the leading death threats nowadays.

### 1.1.2 Bladder Cancer

Bladder cancer refers to any of several types of malignant tumour arising from the urinary bladder which is the organ that collects urine excreted by the kidneys . About 90% of bladder cancers are transitional cell carcinoma (TCC or called urothelial cell carcinoma, UCC). Cancer arises and develops from the cells of the inner surface of the bladder. Among all the cases of various cancers, urinary bladder tumour is ranked as the fourth most common cancer with over 7,000 new cases and the six highest cause of death resulting

in more than 3,400 deaths among British males in 2011. Notably, the risk of developing bladder cancer of men is about 2.5 times higher than that in women.

#### 1.1.2.1 Risk Factor

Risk factors are influences that change the likelihood of developing a disease such as cancer. There are many known risk factors associated with bladder cancer. In other words, bladder cancer is the end result of the variable combination of risk factors. Cigarette smoking is one of the major risk factors for bladder cancer due to the fact that more than 50% of bladder cancer population in men and 30% in women are accounted with smoking habit (Kakehi et al., 2010). Workplace exposures also result in cancer. For example, being around certain chemicals used in the manufacture of dyes, rubber, leather, textiles and paint products increases the risk of bladder cancer. Exposure to arsenic in drinking water at concentrations higher than 300 µg/l is also strongly associated with bladder cancer (Letasiova et al., 2012). In addition, certain industrial chemicals called aromatic amines, such as benzidine and beta-naphthylamine have also been reported to cause bladder cancer (Ferrís et al., 2013; Letasiova et al., 2012). Age and gender are other

risk factors for developing bladder cancer. Bladder cancer occurs more commonly in the elderly population and shows a 4:1 ratio of males to females (Ferrís et al., 2013; Shariat et al., 2010).

The most important of all, variant genetic polymorphisms associate with bladder cancer have been revealed recently. Single nucleotide polymorphism (SNP) in CDKN2A intronic region has been implicated in the pathogenesis of bladder cancer (Li et al., 2014). Genetic variation in TP63C/T, TERTC/T, and SLC14A1C/T were also confirmed associated to bladder tumour in European and White population. Multiple SNPs in base excision repair system including XRCC1 modulate the risk of bladder cancer, which modify survival of bladder cancer patients treated with chemotherapy (Sacerdote et al., 2013; Xie et al., 2013). However, individual prediction of disease outcome based on “molecular markers” of bladder cancer is currently not reliable. Due to reproducibility issues, these markers are not yet used in the daily clinical routine even they are considered to predict progression and survival (but not recurrence) (van Rhijn et al., 2014). Reproducibility of marker assessment and consistency of examine results needs more studies in the future.

#### 1.1.2.2 Bladder Cancer Stage

The stage of cancer is based on how big the tumour is and how far it has spread. The most common system to describe cancer stage is the TNM (Tumour, Node, Metastasis) system which is available for all cancers. T stands for how deeply the tumour has grown. N indicates whether there is cancer found in lymph nodes and M means whether the tumour cells have spread to any other area of body. Combined with the number staging system, the development of cancer is well defined and makes it much easier to decide the correct treatment options. In general, the number staging system is usually classified into four main stages. Stage 1 is the most benign of cancer and stage 4 is the most advanced stage.

##### 1.1.2.2.1 The T stages of bladder cancer (See Fig.1.1)

- T0 – no evidence of a primary tumour
- CIS (also called Tis) – *in situ* carcinoma, tumours are only in the deepest layer of the bladder lining
- Ta – the tumours are non-invasive in the deepest layer of the bladder lining
- T1 – the tumours have started to grow at the connective tissue below the bladder lining

- T2 – the tumours have grown through the connective tissue to the muscle
- T2a – only superficial muscle involvement
- T2b – deep muscle involvement
- T3 – the cancer has extended beyond bladder wall
- T3a – microscopic invasion, the tumours in the fat layer can only be observed by using a microscope
- T3b – macroscopic invasion, the tumours in the fat layer are large enough that they can be observed on image tests, or felt during an examination under anaesthetic by a surgeon
- T4 – the tumours have spread outside the bladder and into nearby organs or structures.
- T4a – the tumours have invaded to neighbouring structures such as prostate, uterus or vagina
- T4b – the tumours have spread to the wall of the pelvis or abdomen (Fig.1.1)

This system is defined according to a combination of the test of cancer cells after a biopsy, inspection of the bladder performed by anaesthetic surgeon and a CT or MRI scanning (*Cancer Research UK*).

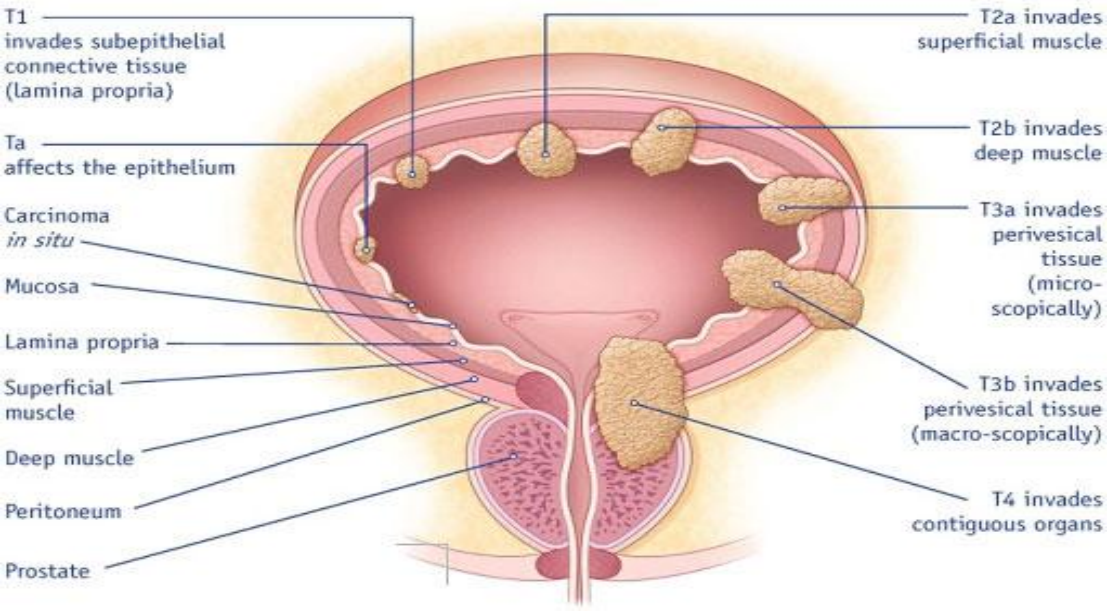


**FIGURE1. 2. The T stage of bladder cancer.** (*Action on Bladder Cancer*)

1.1.2.2.2 The N Stage of Bladder Cancer

The N category describes whether the cancer spreads to the lymph nodes near the bladder. Like the T stage of bladder cancer, there are four lymph node stages defined in bladder cancer as well. CT or MRI scan is able to see and measure the lymph nodes, or they may be found during the surgery for removing bladder.

- N0 – no cancer is found in any lymph nodes
- N1 – tumour is found in only one lymph node in the pelvis
- N2 – tumour is found in more than one lymph node in the pelvis
- N3 – tumour is found in one or more lymph nodes in the groin

#### 1.1.2.2.3 The M Stage of Bladder Cancer

If there is no sign of any cancer invading to another body organ, it marks as M0. In contrast, M1 indicates that the cancer has spread to distant areas of the body. Bones, lungs, liver and distant lymph nodes are the most common sites of bladder cancer metastases.

#### 1.1.2.2.4 The Grade of Bladder Cancer

In addition to cancer stages, the term “tumour grade” means how well the cancer cells developed (*Cancer Research UK*).

- Low grade cancers – they look very much like normal cells and are well differentiated and growing slowly
- Medium grade cancer cells – they look abnormal and are moderately differentiated and more likely to spread into the muscle layer of the bladder or to recur after treatment
- High grade cancer cells – they look extremely abnormal and are high graded and poorly differentiated. These tumours grow quickly, are more likely to recur after treatment and more likely to invade into the muscle of bladder

Additionally, the World Health Organization (WHO) has developed a new classification system for bladder cancer in 2004, which divides the cancers into four categories:

- Urothelial papilloma – the tumour is non cancerous (benign)
- Papillary urothelial neoplasm of low malignant potential (PUNLMP) – the tumour grows very slowly and unlikely to spread
- Low grade papillary urothelial carcinoma – the tumour grows slowly and unlikely to spread
- High grade papillary urothelial carcinoma – the tumour grows more quickly and more likely to spread

#### 1.1.2.3 Treatment of Bladder Cancer

Treatments for cancer of the bladder are generally classified as four main types: Surgery, intravesical therapy, chemotherapy and radiation therapy. Surgery is the main and may be recognised the most successful treatment for most solid tumours including bladder cancer. Recently, the gold standard for the detection of bladder cancer has been considered as conventional or white-light cystoscopy. However, it is difficult to examine flat lesions such as cancer *in situ* with this technique and the disadvantage can further result in

incomplete tumour resection (Jacobs et al., 2010). With intravesical therapy, the medicine is directly administrated into the bladder rather than given by mouth or injected into a vein. This increases the likelihood that only tumour cells in bladder will be affected by medicine. Any cancer cells outside of the bladder or have grown deeply into the bladder wall are not treated. Medicines administrated into the bladder are not able to reach cancer cells in other organs such as kidneys, ureters, and urethra. Currently, radiation and chemotherapy are two major cancer treatments of bladder tumour (Kaufman et al., 2009). Nevertheless, it is a problem that the recurrence is still high (Soloway et al., 2002). Of newly diagnosed bladder cancer cases, although almost a quarter will present with non-muscle-invasive diseases and are not death threatening, however more than half of the patients will show the recurrences and most of those will be within five years (Sylvester et al., 2002). Furthermore, approximately 30% of cases will progress to muscle-invasive cancers and result in life threatening from distant metastases (Saad et al., 2002).

## 1.2 Cell Cycle Regulation and Cancer

### 1.2.1 The Cell Cycle

The cell cycle, or cell-division cycle refers a process of cellular events that occur in a cell associating with its duplication and division, which leads to the production of two daughter cells. The cell cycle consists of four phases $G_1$ (gap), S (synthesis), $G_2$ phase and M (mitosis). In $G_1$ phase, the proteins for DNA replication are synthesized. During S phase, the cell increases its size and DNA replication occurs. G2 is the gap between DNA synthesis and mitosis. Cells continue to grow in this stage. Everything for M phase and cell division is prepared and confirmed ready before entering M phase. Finally, cell growth stops at M phase. Two identical daughter cells are derived from one single cell and re-enter $G_1$ phase again.

### 1.2.2 Cell Cycle Control

Cell cycle progression is an accurate mechanism which is well-controlled by specific holoenzymes made up of the regulatory cyclins and catalytic cyclin-dependent kinases (CDKs). The specific cyclins and corresponding CDKs can form complexes called cyclin-CDK (cyclin-dependent kinases) complexes resulting in the activation of CDKs. In 2001, Timothy R Hunt, Paul

M Nurse and Leland H Hartwell shared the Nobel Prize in Physiology or Medicine for their discoveries of key regulators of the cell cycle (Balter and Vogel, 2001). In mammalian cells, these multiple CDKs and cyclins which regulate passage through the cell cycle can be classified to four main groups by the stage of cell cycle:

- Mid $G_1$: CyclinD-CDK4/6
- Late $G_1$: Cyclin E-CDK2
- S phase: Cyclin A-CDK2
- Mitotic phase: CyclinA/B-CDK1

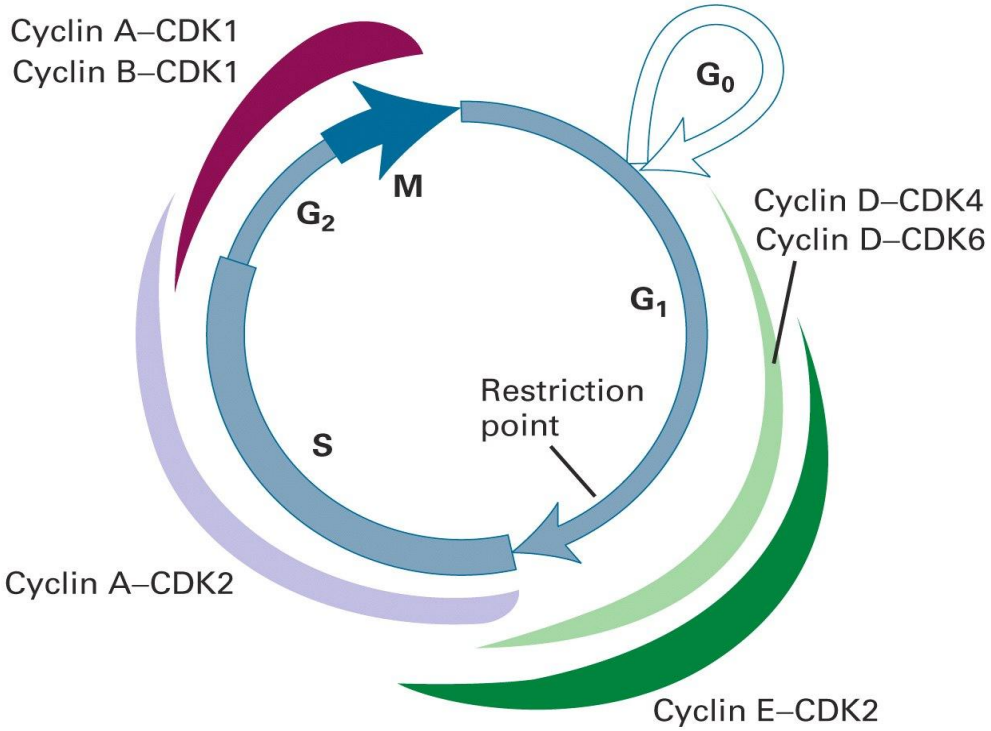


**FIGURE1.3. The cell cycle progression.** Multiple CDKs & cyclins regulate passage of mammalian cells through the cell cycle (taken from *Molecular Cell Biology 5th edition*).

### 1.2.3 Cell Cycle Control and Cancer

As described previously, most cancer cells are derived from normal cells which show many alterations in signal transduction pathways leading to uncontrolled proliferation. Molecular studies have revealed that cell cycle regulators are frequently mutated in many human tumour cells. This indicates the importance of correct cell cycle regulation in cancer prevention. These aberrant regulations of cyclins and CDKs are advantage tumour cells to bypass the normal regulation resulting in uncontrolled cell cycle progression (Malumbres and Barbacid 2001).

### 1.2.4 Cell Cycle Checkpoints

It has been reported from the review article in *Cancer Cell* in 2003 (Bartek and Lukas, 2003) that losing cell cycle control causes accumulation of genetic aberrations which further results in transformation of normal cells to tumour cells and lead to tumourigenesis and carcinogenesis and eventually invasive, malignant cancers. When DNA damage or replication stress occurs, checkpoint systems are activated, which arrests cell cycle progression in order to have time for DNA repair or induce cell apoptosis to prevent the damage becoming worse. In mammalian cells, there are two highly conserved signal

transduction pathways with two key molecules: checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) which participate in checkpoint systems (Fig. 1.3) to guarantee the genome stability and the accuracy of cell cycle progression such as the completed cell mitosis or DNA replication of the entire cell cycle (Bartek and Lukas, 2003).

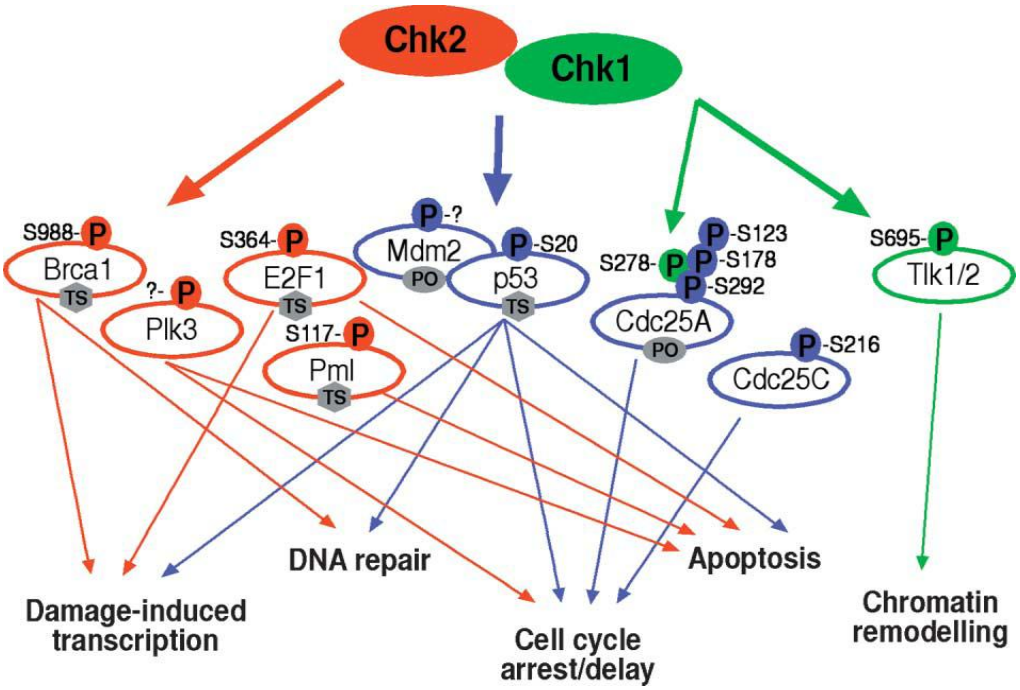


**FIGURE1.4. Two conservative pathways involved in checkpoint systems.** Chk1/2 respectively regulates different downstream proteins such as Mdm2, p53 and Cdc25 family and participates in checkpoint system. Red proteins are regulated by Chk2; Green one by Chk1; Blue ones by both (Bartek and Lukas, 2003).

Chk1 and Chk2 are structurally unrelated serine/threonine protein kinases, with overlapping function in the checkpoint signal transduction pathway. These two protein kinases have already been proven to trigger in response to various

stimuli (Bartek and Lukas, 2001; McGowan, 2002). Activation of Chk1 or Chk2 relies on phosphatidylinositol 3-kinase family particularly ataxia telangiectasia and Rad3-related protein (ATR) and ataxia-telangiectasia mutated protein (ATM) (Abraham, 2001). These upstream PIKK proteins then activate and phosphorylate the two downstream crucial checkpoint kinases respectively. It is well accepted that while the ATM-Chk2 signalling cascade is activated mainly in response to DNA double strands breaks (DSBs), ATR-Chk1 transduction pathway responds to DNA single strand lesions and especially DNA replication stress (Fig. 1.4).

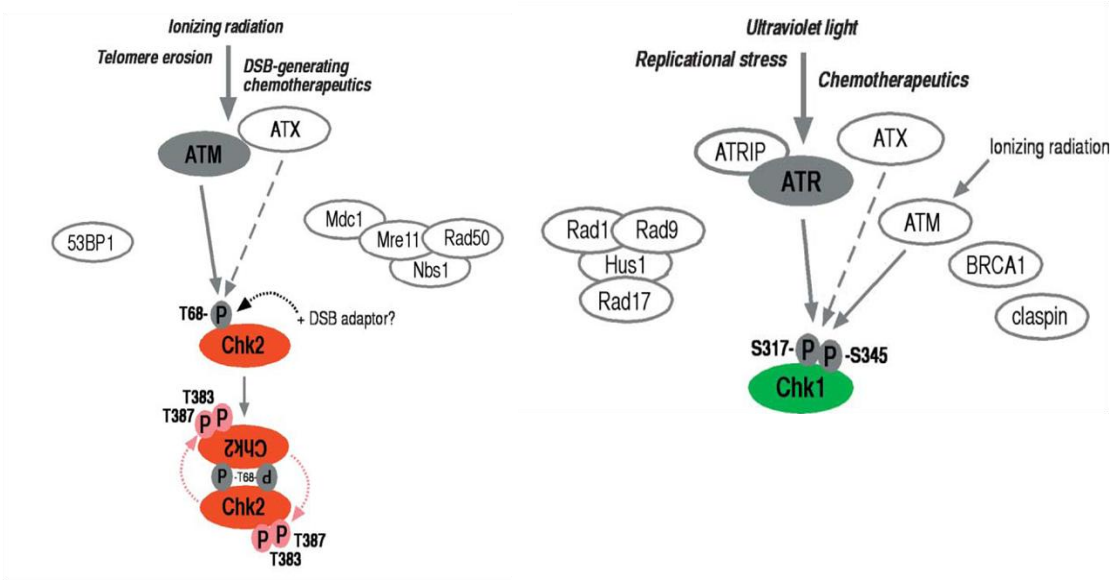


**FIGURE1.5. Two conserved pathways involved in checkpoint systems.** The ATM activates downstream Chk2 by phosphorylating Ser68 in response to DSBs whereas Chk1 is mainly activated by ATR with Ser345, Ser317 phosphorylation following the presence of ssDNA. (Bartek and Lukas, 2003)

For example, ATR was proven to be activated in response to various genotoxic agents and chemicals which stall replication folks such as topoisomerase inhibitors and hydroxyurea (HU) (Melo and Toczyski, 2002).

#### 1.2.4.1 The $G_1$ Checkpoint

Since Chk1 kinase expression is restricted to S-$G_2$ phases of the cell cycle, the major $G_1$ checkpoint is governed by the ATM-Chk2-p53-p21 pathway to confirm all events occurring in $G_1$ phase are correctly operated before entering S-phase (Lukas et al., 2001). DNA damage is detected by ATM which has been demonstrated to be able to activate the downstream tumour suppressor protein p53 (Banin et al., 1998). Activated p53 up-regulates p21 which is an inhibitor of CDK2 (Li et al., 1994) leading to the inhibition of CyclinE/CDK2 and delayed $G_1$ phase progression.

#### 1.2.4.2 The Intra-S Checkpoint

The intra-S checkpoint can be triggered in response to both DNA double strand breaks (DSB) and stalled replication fork-induced single strand DNA (ssDNA). Activated intra-S checkpoint results in Chk1/Chk2 activation leading to Cdc25A phosphorylation and trigger its degradation (Mailand et al., 2000)

which causes CDK2 to remain inactivated and unable to promote cell cycle progression through S phase.

#### 1.2.4.3 The $G_2$/M Transition Checkpoint

The $G_2$/M transition checkpoint prevents mitotic entry in the presence of un-replicated DNA induced from previous cell cycle stage or DNA damages. In response to incomplete DNA replication or DNA lesions, ATR-Chk1 pathway phosphorylates Cdc25C, which targets it for transport out of the nucleus (Xiao et al., 2003) so that CDK1 remains inactivated and terminates $G_2$/M progression.

### 1.2.5 The Two Highly Conserved Checkpoint Pathways

#### 1.2.5.1 Chk1 and Chk2

It is commonly accepted that Chk2 acts as a stable protein which presents throughout entire cell cycle, however Chk1 is an unstable protein that its activation mainly restricts to both S and $G_2$ phase (Lukas et al., 2001). Chk1 kinase plays a role at S-$G_2$/M phase transition of cell cycle even in undisturbed cell cycle in human fibroblasts without DNA damage (Kaneko et al., 1999). In addition, according to studies based on embryonic stem cells, Chk1 was demonstrated to play a crucial role for mammalian development and cell viability. Mice with Chk1 depleted and embryonic cells with Chk1 deficiency were lethal (Liu et al., 2000). However the Chk2-deficient mice were viable with normal features, and exhibited resistance to ionizing radiation (IR) without any enhanced level of tumourigenesis (Takai et al., 2002). Although Chk1 knockout is not lethal in somatic cells, many cell types with Chk1 depletion grow slowly and accompany increased spontaneous apoptosis (Meuth, 2010). Furthermore, during DNA replication stress, siRNA-mediated Chk1 suppression was showed to result in significant cell apoptosis (Myers et al., 2009). These findings indicate the hypothesis that Chk1 plays a role protecting tumour cells from apoptosis induced by DNA replication defects.

### 1.2.5.2 The Mechanism of Chk1 in Cell Cycle Checkpoint

In the past two decades, the potential mechanism of how Chk1 regulates S and $G_2$ phase arrest was established. The checkpoint kinase regulates the activation of downstream Cdc25 phosphatases especially Cdc25A and Cdc25B which remove phosphates from inhibitory tyrosine and threonine residues of cyclin-dependant kinases (CDKs) are essential for cell cycle progression (Furnari et al., 1997; Sanchez et al., 1997). Afterward, the activated cyclin-CDK complex finally contributes cell cycle to further progress (Xiao et al., 2003) (Fig. 1.5).

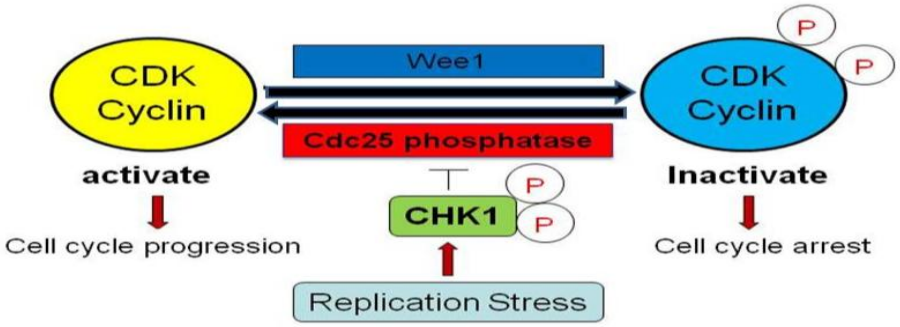


**FIGURE1.6. Schematic representation of the effect of Chk1 on Cyclin-CDK complex regulation.** Replication stress-activated Chk1 regulates CDC25A which removes the inhibitory phosphate from CDK, which promotes cell cycle progression.

Intriguingly, Cdc25A, Cdc25B, but not Cdc25C have already been demonstrated to be over-expressed in many aggressive human cancer cell lines and recognised as proto-oncogenes (Wu et al., 1998). During the past few years, more mechanisms of Chk1 function have been further established.

As described previously, Chk1 is necessary to regulate the activation of replication origins via Cdc25 phosphatases in undisturbed S phase (Shechter et al., 2004). Furthermore, Chk1 has also been found to be required to maintain replication fork progression in vertebrate cells even without DNA damage (Petermann et al., 2006). According to *In vitro* siRNA-based experiments, it was reported that human Chk1 suppression also led to a transient increased initiation of DNA replication and DNA breakage in human osteosarcoma (Syljuasen et al., 2005). Similarly, it was shown that even without external DNA replication defects, the cells treated with the specific Chk1 inhibitor alone also result in a delayed S phase progression in not only normal primary cells but also several cancer cell lines. Additionally, due to the fact that human beings have a large genome, there are approximately 50,000 replication origins that can be used during S phase. From *in vitro* observations of vertebrate cells, Chk1 is also found to modulate the density of replication origin activation by inhibiting latent origin firing during undisturbed S phase progression (Maya-Mendoza et al., 2007). Therefore, it is not surprising that Chk1 kinase acts as a workhorse not only in damaged cells but also in normal undisturbed cells whereas nonessential Chk2 is conditionally activated in response to DNA damage. Altogether, these studies strongly indicate that

aberrant Chk1 function may play a crucial role in the progression and development of tumour transformation.

### 1.2.6 Potential Applications of Chk1 in Cancer Therapy

As described previously, cell cycle progression is a highly controlled process and accurately monitored by surveillance systems such as cell cycle checkpoints. Since Chk1 kinase is over-expressed in many types of tumours (Verlinden et al., 2007), Chk1 seems to play a role to protect cancer cells from cell apoptosis in response to the treatment of anti-cancer drugs. Since tumour cells commonly lack $G_1$ checkpoint and rely on S and $G_2$ checkpoints instead, this incident suggests a potential of novel cancer therapeutics with the combination of Chk1 inhibitors and radiotherapy or clinical anti-cancer drugs. For example, UCN-01 (7-hydroxystaurosporine) is widely recognised as a powerful but unselective Chk1 inhibitor (Graves et al., 2000). It has been reported to sensitize various cancer cells to variant DNA-damaging agents such as cisplatin (Wang et al., 1996), topoisomerase I inhibitors (Shao et al., 1997) or gemcitabine (Shi et al., 2001). However, the usage of UCN-01 on Chk1 inhibition was not totally successful since its untoward toxicities and non-specific inhibition decrease the value as a Chk1 inhibitor (Sausville et al.,

2001). On the other hand, CHIR-124 is a quinolone-based small molecule which selectively inhibits Chk1 *in vitro* (Ni et al., 2006; Tse et al., 2007b). The combination of CHIR-124 and topoisomerase poisons was proven to reduce proliferation in many p53-mutant cell lines (Tse et al., 2007a; Tse et al., 2007b). Furthermore, CHIR-124 was also reported able to induce S and $G_2$/M checkpoints and result in cell apoptosis via the synergistic interaction with the topoisomerase poisons such as camptothecin or SN-38 in MDA-MD-435 breast cancer cells (Tse et al., 2007b). Additionally, CHIR-124 was also shown to enhance the growth inhibitions and apoptosis of CPT-11 in tumour cells by compromising the checkpoint activation (Tse et al., 2007b). However, the development of Chk1 inhibitors in clinical cancer therapy is still a challenge as it is not easy to confirm that the Chk1 activation has been successfully suppressed by the inhibitors. Although the $Ser^{216}$ phosphorylation of Cdc25C in response to DNA damage is widely considered as a marker of Chk1 activation (Peng et al., 1997; Sanchez et al., 1997), the specific site is also constitutively phosphorylated by an unidentified kinase even in undamaged cells. This makes it difficult to be a reliable marker of Chk1 inhibition (Kohn et al., 2002).

The increased expression of γ-H2AX is considered as another downstream marker of Chk1 knockdown in chemotherapy (Abraham, 2004). H2AX was revealed to be phosphorylated at serine 139 (γ-H2AX) in response to Chk1 inhibition particularly when followed by treated with DNA replication inhibitors (Ewald et al., 2007; Syljuasen et al., 2005). Since the increased level of γ-H2AX is recognised as a marker in response to DNA double-strand breaks, it is not surprising that loss of Chk1 kinase activity could result in DNA damage and further cell apoptosis (Carlessi et al., 2009; Durkin et al., 2006). However, instead of playing a direct role in the Chk1 associated signalling transduction pathway, γ-H2AX foci in Chk1-depleted cells were supposed to point out the sites of where persistent replication fork defect occurs (Gagou et al., 2010). Furthermore, as described previously that Chk1 plays a crucial role which ensures genome stability during cell cycle progression, whether secondary cancers will appear due to the genetic instability caused by checkpoint depletion is not clear. There have been many studies demonstrated that inhibition of ATR-Chk1 signalling cascades promotes tumourigenesis in select cancer cell lines (Bric et al., 2009; Fang et al., 2004). Therefore, the potential side effect that the secondary cancers arise due to Chk1 suppression needs more investigation (Tse et al., 2007a).

As described previously, tumour cells develop multiple mechanisms and more rely on DNA repair and checkpoint systems to evade damage-induced cell death. Therefore, it is not surprising that these DNA damage repair and signalling to cell cycle checkpoints (or known as DNA damage response, DDR) are considered as highly potential candidates for targeted therapy against cancer. However, dysfunction of one DNA damage response is sometimes functional compensated by another compensatory DDR pathway which contributes to drug resistance to DNA-damaging chemotherapy or radiotherapy (Curtin, 2012). Therefore, the other very likely reason for the lack of clinical efficacy of Chk1 inhibitors may be that the decrease of Chk1 activation triggers the functional compensation of ATM and ERK1/2 pathways. Thus, using a synthetic lethal approach to selectively kill tumour cells which are dependent on a compensatory DDR in response to damage-caused stress for survival is currently becoming a hot issue. For example, suppression of many enzyme activities, non-essential components of cell cycle regulation may disturb Chk1 inhibitor–induced ERK1/2 activation and further enhance the toxicity of Chk1 inhibitors (Dent et al., 2011). So far, there are still several CHK1 inhibitors which are undergoing clinical trials. The potent Chk1/Chk2 inhibitor AZD7762 is under phase I, dose-escalation study to evaluate the

safety, pharmacokinetics (PK) and preliminary efficacy alone or in combination with gemcitabine in Japanese patients with advanced solid malignancies (Seto et al., 2013). Similarly, other CHK1 inhibitors including PF-477736 (Pfizer) and SCH900776 (Schering Plough) are also under Phase I or II clinical evaluation in patients (Ashwell et al., 2008; Karp et al., 2012). More Chk1 inhibitors applied on clinical trials are listed as a table below:

| Compound | Known Targets | Combination Studies | Clinical trials |
|---|---|---|---|
| UCN-01 | PKC family (Mizuno et al., 1995)<br>Chk1 (Busby et al., 2000) | Mytomycin C (Akinaga et al., 1993)<br>Cisplatin (Bunch and Eastman, 1996)<br>Topoisomerase I poisons (Tse et al., 2007a)<br>Gemcitabine (Shi et al., 2001) | Phase I/II |
| Gö6976 | PKCα (Martiny-Baron et al., 1993)<br>Chk1 (Jia et al., 2009)<br>TrkA/B (Behrens et al., 1999)<br>JAK2 (Grandage et al., 2006) | Doxorubicin (Aaltonen et al., 2007)<br>Cisplatin (Qamar et al., 2008)<br>Radation (Sidi et al., 2008)<br>Etoposide (Huigsloot et al., 2003) | n/a |
| CHIR-124 | Chk1 (Tse et al., 2007a; Tse et al., 2007b) | Topoisomerase 1 poisons (Tse et al., 2007b) | Preclinical |
| AZD7762 | Chk1/2 (Dent et al., 2011) | Gemcitabine (Ashwell et al., 2008; Seto et al., 2013) | Phase I |
| PF-477736 | Chk1 (Dent et al., 2011) | Gemcitabine (ClinicalTrials.gov) | Phase I |
| SCH900776 | Chk1 (Dent et al., 2011) | Gemcitabine (Daud et al., 2010)<br>cytosine arabinoside (Karp et al., 2012) | Phase I/II |

### 1.2.7 Replication Protein A (RPA)

When replication stress occurs, RPA (Replication Protein A or also known as replication factor A, RFA) quickly coats single-stranded DNA and recruits ATR-CHK1 cascades to trigger checkpoint activation. RPA is a stably crucial protein complex which consists of three subunits: RPA14, RPA32 and RPA70 with about 14, 32 and 70kDa respectively (Wold and Kelly, 1988). It has been shown to be involved in almost all kinds of cellular DNA metabolism. Activated RPA participates in not only DNA replication, recombination or transcription but also in many various DNA repair mechanisms such as base excision, nucleotide excision, mismatch and DNA double-strand break repair (Wold, 1997; Zou et al., 2006). The most common feature of RPA is its single-strand binding protein (SSB). In contrast to double-stranded DNA or RNA, RPA complex has shown a much higher binding affinity to single-stranded DNA (Kim et al., 1992). More than 25 years ago, RPA was firstly purified from human HeLa cell extracts in which RPA acts as a component of DNA replication for simian virus 40 (SV40) *in vitro* (Wobbe et al., 1987). While the genes of RPA32 and 70 subunit are highly conserved in human RPA complex, the smallest RPA14 subunit has a much lower conservation level across different species (Wold, 1997). It is well accepted that RPA32 and RPA14 act

as essential precursors to the proper folding of entire RPA complex whereas RPA70 is reported to serve as a DNA binding activity (Henricksen et al., 1994; Wold et al., 1989). Since RPA serves as a necessary regulator in DNA metabolism, it is not surprising that RPA has primarily been found to localise in the nucleus and forms foci during DNA replication (Brush et al., 1995). Furthermore, the mRNA expression of RPA was also shown to co-ordinate with the cell cycle progression particularly during S phase in both S. *cerevisiae* and C. *fasciculatea* (Brill and Stillman, 1991; Pasion et al., 1994). RPA32 subunit is phophorylated at the conserved cyclin-dependent kinase (CDK) phophorylation sites Ser23 and Ser29 by variant protein kinases including multiple CDK family and Ser4/8 by DNA-dependent protein kinase (DNA-PK) during $G_1$/S phase transition and in M phase in undisturbed cell cycle passage (Pan et al., 1994; ZernikKobak et al., 1997). Furthermore, RPA32 can also undergo further hyper-phophorylation at Ser4. Ser8 Ser11/12/13, Thr21, Ser23, Ser29 and Ser33 following various kinds of DNA damage (Nuss et al., 2005). This DNA damage-triggered RPA hyper-phophorylation is considered to be activated by phosphatidylinositol 3-kinase-related kinases (PIKKs) family including ataxia-telangiectasia mutated (ATM), ataxia- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Block et al., 2004).

Unfortunately, full details of the role RPA hyper-phosphorylation plays in response to DNA damage is still not clearly understood. Although RPA was originally considered as a single-stranded DNA binding protein, it is also found able to interact with other proteins. For instance, RPA was reported to play a necessary role for chromatin association with ATR following DNA strand breaks (You et al., 2002). Intriguingly, RPA was also found to act as a sensor for ATR recruitment at DNA damage sites and further trigger downstream Chk1 activation (Ball et al., 2005; Zou and Elledge, 2003). In addition, RPA has been reported to be associated with cancers. Up-regulated RPA expression was found in several types of tumours including breast and colon cancers and has been demonstrated to play a crucial role in cell proliferation during tumour growth and progression (Givalos et al., 2007; Tomkiel et al., 2002). L221P mutation in the high-affinity DNA binding site of RPA70 subunit has been reported to result in defects in checkpoints and DNA repair in yeast (Chen and Kolodner, 1999; Chen et al., 1998) and embryonic lethality in mice when homozygous (Wang et al., 2005). Even in heterozygous mutant embryonic fibroblasts, the mutation in DNA binding subunit of RPA70 results in shortened life spans and elevated cancer rates in mouse (Wang et al., 2005). In contrast to RPA70, hyper-phosphorylation of RPA32 was shown to facilitate mitotic exit

following mitotic DNA damage. When endogenous RPA2 was replaced by a mutant subunit, cells were unable to complete mitosis and release early from M phase into an abnormal 2N $G_1$ phase (Anantha et al., 2008). Furthermore, foci of RPA2 hyper-phosphorylation have also been demonstrated to form and dramatically increase in Chk1-depleted colon cancer cells in response to DNA replication inhibitor in advance of the induction of apoptosis (Rodriguez et al., 2008). Taken together, these studies strongly suggest that RPA indeed plays a crucial role in DNA damage-induced cell cycle checkpoint mechanism.

## 1.3 Aims and Hypothesis

During the past decade, the role of Chk1 has been well established and reported to participate in tumourigenesis by several studies around the world. However, the cell models examined in Chk1 studies were either tumour cell lines or normal fibroblasts. The role of Chk1 in normal epithelial cells is still poorly understood. Due to the fact that most solid tumours show epithelial origins, it is worthwhile to investigate the role of Chk1 in normal epithelial cells. siRNA-mediated Chk1 depleted cells have been reported to increase cell apoptosis particularly in response to DNA replication defects. This strongly suggests that ATR-Chk1 plays a role in protection from cell death under DNA replication stress and provides a valuable course for targeted therapy. Here I hypothesise that malignant cancer cells rely upon ATR-Chk1 checkpoint for survival, however this dependence may not appear in normal epithelial cells. The aim of this study is to determine the role of Chk1 especially in normal human urothelial cells under DNA replication stress. Through the work to define the role of checkpoint signalling transduction cascades in regulating DNA replication stress-induced cell cycle aberration in both normal and tumour cells will be possible to identify potential mechanisms of cancer transformation and able to plan novel managements for bladder cancer therapy.

# CHAPTER 2 MATERIALS AND METHODS

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# Chapter 2 Materials and Methods

## 2.1 General laboratory equipments and materials

### 2.1.1 General laboratory equipment

#### 2.1.1.1 Laboratory Equipment

| Equipment | Supplier |
|---|---|
| 7900HT Fast Real-Time PCR System | Applied Biosystems |
| CellQuest FACS Analysis Software | CellQuest |
| Class II Biological Safety Cabinet | Walker Safety Cabinets Ltd |
| $CO_2$ Incubator MCO175 | Sanyo |
| Electronic Pipetting Aid | Drummond Scientific Co |
| Eppendorf 5415D Centrifuge | Eppenorf |
| Eppendorf minispin Centrifuge | Eppendorf |
| FACsCalibur | Becton Dickinson |
| Gel Electrophoresis Unit (WB) | Bio-Rad |
| Ice Machine | Scotsman Ice Machines |
| ImageJ Image Processing Software | National Institutes of Health |
| LAS-3000 Chemiluminescence Imaging System | Fujifilm |

| | |
|---|---|
| Mistral 3000i Centrifuge | Sanyo |
| MULTISKAN FC | Thermo SCIENTIFIC |
| P10, P20, P200 and P1000 Pipettes | Starpet |
| Power Pack 300 | Bio-Rad |
| Transfer tank | Bio-Rad |
| PTC 200 DNA engine | GRI |
| BBA tube Heater | Grant Boekel |
| RNA quantification (Nano Vue plus) | BIOCHROM LTD |
| SDS 2.2.1 real-time PCR Analysis Software | Applied Biosystems |
| SPSS Statistics | IBM Corporation |
| The vortex machine | SCIENTIFIC INDUSTRIES, INC |
| Waterbath | Labinco |

#### 2.1.1.2 Laboratory Consumables

| Item | Supplier |
|---|---|
| 15ml Centrifuge tubes | Sarstedt |
| 50ml Centrifuge tubes | Corning Inc |

| | |
|---|---|
| 6-well plates | Cellstar |
| 10cm Petri dish | Greiner bio-one |
| 384-well PCR plates | Life Technologies |
| 75cm$^2$ Vented culture flasks | Nunc |
| 5ml, 10ml and 25ml Stripettes | Corning Inc |
| 500 l and 1.5ml microfuge tubes | Sarstedt |
| Gilson pipette tips | Sarstedt |
| Gloves | Schottlander |
| Syringes | BD Biosciences |
| Tissue Culture Flask | FALCON |

### 2.1.2 Laboratory Chemicals

| Item | Supplier |
|---|---|
| Acrylamide 92.6) 30% Protogel | Geneflow #EC-890 |
| Ammoium Persulphaten | VWR Merck #443074F |
| B-mercaptoethanol | Sigma-Aldrich #M6250 |
| Bovine Serum Albumin (BSA) | Fisher Scientific #BP1600-100 |
| CHIR-124 | SelleckChem #S2683 |
| DMSO | Sigma-Aldrich #101402050 |
| Ethanol | FisherChemical #E/0650DF/17 |
| Gemcitabine | Sigma-Aldrich #G6423 |
| Gö6976 | Sigma-Aldrich #G1171 |
| Hydrochloric Acid | VWR Merck #30024.290 |
| Hydroxyurea | Sigma-Aldrich #H8627 |
| KU55933 | Calbiochem #118500 |
| KU60019 | Selleckchem #S1570 |
| Methanol | FisherChemical #M/4450/17 |
| MG132 | Selleckchem #S2619 |
| N-Acetylcysteine | Sigma-Aldrich #A0737 |

| | |
|---|---|
| Nocodazole | Sigma-Aldrich #M1404 |
| PhosStop Phosphatase inhibitor | Roche #04906837001 |
| PMSF | Sigma-Aldrich #P7626 |
| Propidium Iodide | Sigma-Aldrich #P4170 |
| SDS | BIOLINE #T5-111G |
| Soybean trypsin inhibitor | Sigma-Aldrich #T6522 |
| TEMED | FisherBioreagent #BP150-20 |
| Thymidine | Sigma-Aldrich #T9250 |
| Tris-base | FisherBioreagent #BP152-1 |
| Triton X-100 | Sigma-Aldrich #X100 |
| Tween 20 | Sigma-Aldrich #P2287 |

#### 2.1.2.1 Mechanism of action of Drugs

Thymidine: Increased thymidine inhibits ribonucleotide reductase by elevated dTTP or dGTP resulting in a deficiency of dCTP pool and acts as a DNA replication inhibitor to synchronize the cells in S phase (Fox et al., 1980).

Hydroxyurea: hydroxyurea, also called as hydroxycarbamide decreases the production of deoxyribonucleotides (Slater, 1973) by suppressing the enzyme activity of ribonucleotide reductase (Platt, 2008). In this study, hydroxyurea served as an inducer of DNA replication stress.

Gö6976:

(12-2(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole, M.W. 378.4) Works as a Chk1 inhibitor and inhibits the Chk1 Ser296 autophosphorylation (Kohn et al., 2003).

CHIR-124:

[(*S*)-3-(1*H*-benzo[*d*]imidazol-2-yl)-6-chloro-4-(quinuclidin-3-ylamino)quinolin-2(1*H*)-one] is a quinolone-based small molecule which potently and selectively inhibits Chk1 in vitro (Ni et al., 2006; Tse et al., 2007b).

KU55933: (2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one) an ATP-competitive inhibitor of ATM kinase (Hickson et al., 2004).

KU60019: KU60019 is an improved inhibitor of ATM kinase from KU55933, which is reported 10-fold more effective than previous one in human glioma cells (Golding et al., 2009).

Gemcitabine:

(4-amino-1-(2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranosyl)pyrimidin-2(1*H*)-on 2′,2′-difluoro-2′-deoxycytidine) is a nucleoside analogue which prevents cells from making DNA and RNA. During DNA replication, gemcitabine replaces the building block, cytidine, resulting in replication termination and apoptosis as only one additional nucleoside can be attached to the defect nucleoside. Furthermore, gemcitabine also binds to ribonucleotide reductase (RNR) and inactivates the enzyme irreversibly, which blocks deoxyribonucleotide formation for DNA replication and repair leading to cell apoptosis (Cerqueira et al., 2007). Currently, gemcitabine has been approved as an anti-tumour drug and applied in clinical cancer therapy to treat various solid carcinomas such as bladder, breast, pancreatic and non-small cell lung cancer (Toschi et al., 2005).

Nocodazole: (Methyl (5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl) serves as a mitosis inhibitor which interferes with microtubule polymerization of mitotic spindle formation and arrest the cell cycle progression at M phase (Lee et al., 2004; Sentein, 1977).

N-Acetylcysteine (NAC): NAC is a derivative of cysteine. It is also a pharmaceutical drug and nutritional supplement. Acetylcysteine serves as a precursor of cysteine which participates in glutathione generation preventing damage caused by reactive oxygen species such as free radicals and peroxides (Pompella et al., 2003). Hence, N-acetylcysteine is commonly considered as a biologic antioxidant reagent. In this study, NAC was used as a ROS inhibitor (Ito et al., 2004).

### 2.1.3 Standard Solutions

i. Purified Water

Double-distilled water ($ddH_2O$) was generated by Purite Prestige Labwater 250 purification system. In this thesis, the term “water” was referred to this ultra-pure water unless stated separately.

ii. Phosphate Buffered Saline (PBS)

PBS was simply prepared by dissolving 10 PBS tablets in 1 litre of water and autoclaved. 1 litre PBS buffer contains 8g NaCl, 0.2g KCl, 1.15g $Na_2HPO_4$ and 0.2g $KH_2PO_4$. The pH was maintained at 7.4.

iii. Trypsin-EDTA

Trypsin is a common reagent which detaches adherent cells from petri-dish, cell culture flasks and each other. In this study, trypsin-EDTA is purchased from Lonza.

iv. PBS Tween 20

PBS Tween20 (PBST) is a solution mixed of Phosphate Buffered Saline (PBS), 0.1% BSA and 0.2% Tween 20, used to increase permeability of cell membrane.

v. TBS Tween20

TBS Tween20 (TBST) is a mixture of Tris-Buffered Saline (a buffer used for

maintaining pH) and Tween 20, which used for washing nitrocellulose membrane in immunoblotting.

### 2.1.4 Mammalian Cell Lines and Primary Cells

In this study, four different cell types were used for research models, see table below.

| Cell | Tissue | Type | Supplied by / Reference |
|---|---|---|---|
| NHU | Bladder | Normal, Primary | Dr. James Catto, (Catto et al., 2009) |
| hTERT-NHU | Bladder | Normal, hTERT-immortalized | Prof. Margaret Knowles (Catto et al., 2009) |
| EJ | Bladder | Grade IV, Invasive, p53 mutated | ATCC |
| TCC-sup | Bladder | Grade IV, transitional cell carcinoma, p53 mutated | ATCC |
| RT4 | Bladder | transitional cell papilloma, p53 wild-type | ATCC |
| RT112 | Bladder | Grade I, p53 wild-type | ATCC |

| | | | |
|---|---|---|---|
| SW780 | Bladder | Grade I transitional cell carcinoma, p53 wild-type | ATCC |
| S637 | Bladder | Grade II, p53 wild-type | ATCC |

# 2.2 Materials and Methods

## 2.2.1 Cell Culture

### 2.2.1.1 Cell Culture Mediums

i. hTERT-NHU (Normal Human Urothelial) and primary NHU cells

I analyzed both immortalized and primary urothelial cells respectively in this work. The former were generated by the transfection of non-immortalized urothelial cells (NHU) with human Telomerase Reverse Transcriptase (hTERT) (gift of Prof. Margaret Knowles, University of Leeds, UK). NHU cells are derived from normal urothelial biopsies (Catto et al., 2009). Both primary and NHU-Tert cells were maintained in Keratinocyte Serum-Free Medium (KSFMc, purchased from GIBCO) supplemented with EGF, BPE and cholera toxin. Purchased EGF and BPE were firstly defrosted in room temperature and then added into KSFMc medium directly with 25μl cholera toxin.

ii. Malignant Bladder Tumour Cells

For comparison, I also analyzed six urothelial carcinoma lines, EJ, TCC-sup, RT4, RT112, SW780 and S637 (purchased from the ATCC**)**. With exception of RT4 and S637, malignant cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% non-essential amino-acids (NEAA). FCS was supplied by Perbio and

stored at -20℃ in 50ml aliquots. Before being added to the DMEM medium, FCS was dialyzed in advance to remove deoxynucleosides that may affect the response to subsequent drug treatments. While RT4 was grown in McCoy culture medium supplemented with 10% fetal calf serum (FCS), S637 was cultured with RPMI medium plus 10% FCS. The preparation of FCS was the same as with other tumour cell lines which were described previously.

#### 2.2.1.2 Cell Culture Procedure

i. Malignant Bladder Tumour Cells

Tumour cells were maintained with DMEM in a 37℃ incubator with 5% $CO_2$ levels. Once these cells were observed to be 90 to 100% confluent or the colour of medium was changed due to pH alteration in response to cell secretions or cell death, they were split at a 1:8 ratio. Culture medium was firstly removed. In order to avoid any possible interference of divalent cations or proteins, cells were then washed with PBS and trypsinized with 1ml trypsin-EDTA in 37℃ for about 5 minutes to be detached. Fully detached cells were harvested with fresh DMEM medium and cultured in new petri-dishes in the incubator.

ii. Normal Human Urothelial (NHU) cells

Both immortalized hTERT-NHU and primary NHU cells were maintained with KSFMc medium in 37℃ supplied with 5% $CO_2$ like tumour cells described previously. The procedures of cell passage were generally the same as tumour cells that mentioned previously except without PBS washing. Notably, the medium used for detached cells collection must be augmented with trypsin inhibitor (50μl in 5ml medium).

### 2.2.2 Western Blot

In this study, protein activation and total protein levels were analyzed by western blot (or called immunoblot). It is a common technique to detect protein levels by different molecular weights of each protein in total protein lysate and widely used in molecular biology, biochemistry or immunology. Since antibody is able to recognize its specific protein antigen, proteins are firstly separated by polyacrylamide gel electrophoresis (PAGE) and probed by antibodies. According to the intensity and the position of blots, the signal of protein expression in cells is capable of analysis.

#### 2.2.2.1 Drug Treatment and Protein Extraction

$5\times10^6$ cells were seeded into 6-well culture dish for 24hr for cell attachment. On the day for drug treatment, cells were refreshed with 37℃ pre-warmed fresh medium and treated with drugs (eq. thymidine, hydroxyurea, Chk1 inhibitor or ATM inhibitor, etc.) for appropriate time points. Afterwards, medium was removed and cells were carefully rinsed with PBS for 4 times. 40μl lysis buffer which contains 25mM Tris-HCl (pH 8.0), 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1M PMSF (phenylmethylsulphonyl fluoride), Protease inhibitor mix, Phosphatase inhibitor mix and

ß-mercaptoethanol were used to lyse cells. Cell lysates were collected into 1.5ml microtube and cooled down on ice for 15minutes with vortex in every 5 minutes followed by a 5 minutes max-speed centrifugation. Supernatant suspension (total proteins) was then transferred to a new 1.5ml eppendorf and kept in -80℃ freezer for later protein quantification.

### 2.2.2.2 Protein Quantification

A protein standard curve and protein samples were prepared as the table below.

| | | BSA 1 mg/mL | RIPA buffer | Sample | H2O | Bradford reagent |
|---|---|---|---|---|---|---|
| | Blank | - | 2 μL | - | 798 μL | 200 μL |
| standart curve | 2.5 | 2.5 μL | 2 μL | - | 795 μL | 200 μL |
| | 5 | 5 μL | 2 μL | - | 793 μL | 200 μL |
| | 10 | 10 μL | 2 μL | - | 788 μL | 200 μL |
| | 20 | 20 μL | 2 μL | - | 778 μL | 200 μL |
| | 40 | 40 μL | 2 μL | - | 758 μL | 200 μL |
| | 80 | 80 μL | 2 μL | - | 718 μL | 200 μL |
| | Sample | - | - | 2 μL | 798 μL | 200 μL |

The absorbance of each solution was measured by photometer at wavelength $OD_{595nm}$ and resulting in a standard curve ($R^2 > 0.98$). According to this, protein concentration can easily be calculated by inner interpolation against the standard curve.

2.2.2.3 Polyacryamide Gel Electrophoresis

Polyacryamide gels were prepared as the table below.

| SEPARATING GEL | 15.00% | 12.00% | 10.00% | 8.00% | 6.00% |
|---|---|---|---|---|---|
| 30% polyacrylamide | 10 mL | 8 mL | 6.7 mL | 5.3 mL | 4 mL |
| 1 M Tris pH 8.8 | 7.5 mL | 7.5 mL | 7.5 mL | 7.5 mL | 7.5 mL |
| 10% SDS | 0.2 mL | 0.2 mL | 0.2 mL | 0.2 mL | 0.2 mL |
| H2O | 2 mL | 4 mL | 5.3 mL | 6.7 mL | 8 mL |
| 10% APS | 0.250 mL | 0.250 mL | 0.250 mL | 0.250 mL | 0.250 mL |
| TEMED | 0.025 mL | 0.025 mL | 0.025 mL | 0.025 mL | 0.025 mL |
| | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL |

While stacking gel is made to stack all the samples of different lanes at the

| STACKING GEL | 5.00% |
|---|---|
| 30% polyacrylamide | 1.7 mL |
| 1 M Tris pH 6.8 | 1.25 mL |
| 10% SDS | 0.1 mL |
| H2O | 6.8 mL |
| 10% APS | 0.125 mL |
| TEMED | 0.0125 mL |
| | 10 mL |

same starting line due to its low pH (6.8) and percentage, resolving gel separates varied proteins by their sizes. The polyacryamide percentage of the gel is directly related to how difficult it is for proteins to pass through the gel during the process of electrophoresis. Within the higher percentage of gel, the lower mobility is shown of proteins. Hence, different proteins with varied molecular weights were fractionated with different concentration of polyacryamide gels. For instance, proteins with large molecular weight (> 200KDa) such as ATM and DNAPK are recommended to be fractionated in 6% gels. Prepared gel solution was poured between two glass plates and set on Bio-Rad electrophoresis system. Protein samples were mixed with 2X protein

loading buffer at 1:1 ratio and heated at more than 95℃ for 5 minutes to denature protein's higher structure folding and loaded into settled gels. The system is run at constant 35mA/gel for 2hr with TGS running buffer.

#### 2.2.2.4 Protein Transfer

In this study, nitro cellulose membrane was used as a carrier for protein transfer from the gels. Protein will be bound to this specific membrane by hydrophobic interactions, as well as charged interactions as its denatured polypeptide form and keeps the bioactivity unchanged. Once running of electrophoresis was finished, the gel could be removed from the apparatus and prepared for later protein transfer. The stacking gel was discarded. Membrane, resolving gels and blotting papers were incubated with transfer buffer (diluted from 100ml 10X transfer buffer with 200ml methanol and 700ml water) for half an hour on shaker. Afterwards, the sandwich cascade was set as blotting paper → gel → membrane → blotting paper from electric current negative to positive and put into transfer tank full with transfer buffer at 200mA for 90 minutes. Transferred membrane was then blocked with TBST containing 5% skim milk for at least 1hr under room temperature on shaker.

2.2.2.5 Probing for Proteins of Interest

Following protein transfer, blocked membrane was then incubated at 4℃ overnight with specific first antibodies. These antibodies were diluted with 5% skim milk which is the same as blocking at appropriate concentration. The rabbit monoclonal antibody against β-actin was used as an internal control.

First antibodies in this study are listed as a table below:

| Antibody | Dilution | Source |
|---|---|---|
| p-Chk1 S296 | 1:500 | #2349S, Cellsignaling |
| p-Chk1 S317 | 1:500 | #2344S, Cellsignaling |
| p-Chk1 S345 | 1:500 | #2348S, Cellsignaling |
| Chk1 | 1:1000 | #2360, Cellsignaling |
| p-ATM S1981 | 1:3000 | #2152-1, EPITMIC |
| ATM | 1:1000 | #K0909, SantaCruz |
| p-Chk2 T68 | 1:500 | #2661, Cellsignaling |
| Chk2 | 1:1000 | Ab8108, abCam |
| P53 | 1:1000 | #9282S, Cellsignaling |
| p-P53 S15 | 1:500 | #9284S, Cellsignaling |
| RPA | 1:1000 | NA19L, Calbiochem |
| p-RPA2 S33 | 1:1000 | A300-246A, BETHYL |
| p-RPA2 S4/8 | 1:1000 | A300-245A, BETHYL |
| γ-H2AX | 1:500 | #2577, Cellsignaling |
| Active-caspase3 | 1:1000 | Ab2302, abcam |
| Cyclin D1 | 1:1000 | #2926, Cellsignaling |
| Cyclin E | 1:1000 | #4129S, Cellsignaling |
| CDK6 | 1:1000 | #3136S, Cellsignaling |
| CDK4 | 1:1000 | sc-260, Santa Cruz |
| P16 | 1:1000 | Ab54210, abcam |
| P19 | 1:1000 | sc-56334, Santa Cruz |
| P21 | 1:1000 | #2947S, Cellsignaling |
| p-RB S780 | 1:1000 | #9307S, Cellsignaling |
| LC3B | 1:1000 | #2775S, Cellsignaling |

| | | |
|---|---|---|
| c-Myc | 1:1000 | #9402S, Cellsignaling |
| β-actin | 1:3000 | A5060, Sigma |

After this the membrane was washed four times, 5 minutes each with TBST and incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies. The enzyme HRP was firstly found in horseradish and widely used in research as it is able to amplify a weak signal and increase the sensitivity of detection of the specific target while reacted with some certain substrates. Here I performed the HRP-conjugated immunoblotting with Enhanced chemiluminescence (ECL) system. During the process of the enzyme catalysed the oxidation of substrates, the reaction emitted low intensity of light. However, the light will be increased up to 1000-fold in the presence of some specific molecules such as p-iodophenol, a modified phenol. (Kapeluich et al., 1997; Kim et al., 1991). In this study, the emitted light was detected and analysed by LAS-3000 programme.

The table of secondary antibodies

| Antibody | Dilution | Source |
|---|---|---|
| Anti-mouse IgG | 1:1000 | #7076S, Cellsignaling |
| Anti-rabbit IgG | 1:1000 | #7074S, Cellsignaling |

### 2.2.3 Flow Cytometry analysis

Flow Cytometry, or called FACS (Fluorescence Activated Cell Sorting) is a biological technique based on laser which beams passing fluid stream resulting the specific light scattering and fluorescence of cells. FACS is widely applied in various fields of biological research such as cell counting, cell sorting and biomarker detection by analyzing the pre-labelled fluorescence signal on each single cell or bio-molecule in a stream of fluid passing through the electronic detection apparatus. In this study, FACS was employed to examine cell cycle alteration, cell death and status of DNA replication in response to some specific treatments.

#### 2.2.3.1 Sample Preparation

In total, $5\times10^5$ cells were seeded into 6-well culture dish for 24 hours to allow attachment. After treatment, medium containing floating cells was firstly collected into 15ml tube. Cells were then washed with 1ml PBS and trypsinized with 1ml trypsin-EDTA in 37℃ for about 5 minutes to be detached. Fully detached cells were harvested with those pre-collected medium and centrifuged at 1000rpm (here equal to 179g) for 3 minutes to remove suspension. Cell pellet was then washed with 1ml PBS at 1000rpm for 3

minutes again and fixed with 70% ethanol to increase the permeability for later propidium iodide staining. Fixed cells were finally stored in -20℃ freezer and ready for later experiments.

2.2.3.2 Cell Cycle analysis (PI, Propidium iodide)

On the day of FACS analysis, ethanol-fixed samples were centrifuged at 1000rpm (here equal to 179g) for 3minutes and washed 2 times with 2ml PBS to remove ethanol. Afterwards, RNaseA was mixed gently with samples to eliminate the possible interferences of RNA and stained with Propidium iodide (PI) for at least 30 minutes. PI is a common intercalating agent and a fluorescent molecule which is capable of inserting into DNA double strands. Once PI is inserted into nucleic acid strands, its red fluorescence is enhanced 20- to 30-fold to enable detection. Since the binding of PI between nucleic acids reflects the amount of DNA directly and due to the fact that DNA amount changes during cell cycle progression, Propidium iodide is commonly used for detecting DNA content in cell cycle analysis. Here I used PI staining with FACS analysis to examine the population for cell death as small DNA fragments occurs during the late stage of apoptosis and cell cycle alteration in response to our specific treatments.

2.2.3.3 Detection of BrdU incorporation in S-phase cells

Fixed cells were washed twice with 2ml PBS and pelleted at 1300rpm (here equal to 179Xg) for 3 minutes and then re-suspended in 1ml 2M HCl to denature DNA double-strands and to allow detection of incorporated BrdU. Acid was added drop by drop to the cell pellet with simultaneously vortexing. After 30 minutes room temperature incubation, samples were spun at 1300rpm for 3 minutes and washed twice with 2ml PBS plus once with PBST (PBS+0.1%BSA+0.2% Tween20) to remove acid and make cells permeable. In 100μl PBST, I added 2μl mouse anti-BrdU (DakoCytomation, M0744) as 1/50 dilution for 20 minutes incubation in dark. After that, cells were spin-down, washed with 2ml PBST again and incubated with rabbit anti-mouse FITC conjugated antibody (DakoCytomation, M0313) as 1/10 dilution in dark for 20 minutes. Finally, secondary antibody bound cells were spun down and washed with 2ml PBS for staining with about 400μl PI solution and RNaseA as described in previous section.

Both PI stained and BrdU incorporated cells were analysed with CellQuest programme (BD Biosciences).

### 2.2.4 Real-Time PCR

PCR (Polymerase Chain Reaction) is also a widely used technique to amplify a single copy of a gene or a particular DNA sequence to thousands or millions of copies. This reaction relies on semi-conservative model of DNA replication of ssDNA and various DNA states under different temperatures. In general, while DNA double strands denature and anneal again at 95℃ and around 60℃ respectively, the thermal-stable DNA polymerase works at about 72℃. Hence, during PCR progresses, DNA samples undergo successive cycles of heating and cooling to let DNA double strains repeated melting and enzymatic replication. However, products of traditional PCR can only be analysed by gel electrophoresis after the full cycles are completed. It is difficult to make a comparison between the level of samples of interest and control gene once the PCR reaction reaches plateau period. On the other hand, real-time PCR, or called quantitative PCR (qPCR) is based on the general principle of standard PCR and able to record DNA replication products in the end of every cycle in “real-time” by detecting non-specific fluorescent dyes or pre-labelled specific probes. This allows more accurate and stable quantification of the increased DNA fragments.

#### 2.2.4.1 RNA Extraction and Quantification

In this study, RNA extraction was performed by GenElute Mammalian Total RNA Miniprep Kit. Treated cells were firstly washed and harvested into 15mL tubes as the standard procedure described previously. Centrifuged pelleted cells were then lysed by the Lysis Solution/2-ME Mixture and thoroughly vortexed until all clumps disappeared. To remove cellular debris and fragmented DNA, cell lysate was put into a GenElute Filtration Column and centrifuged at max speed for 2 minutes. After that, an equal volume of 70% ethanol solution was added and mixed thoroughly to the filtered lysate. Maximum 700 μL of the lysate/ethanol mixture was put into a GenElute Binding Column for a 15-second maximum speed centrifugation. The binding column was then returned to the collection tube and any remaining lysate/ethanol mixture was applied to the column. The column was washed with 500 μL Wash Solution 1 by a 15-seconds maximum speed centrifugation. The binding column was then transferred into a fresh 2 ml collection tube and washed two times with 500 μL ethanol-diluted Wash Solution 2 with another 15-second maximum speed centrifugation. The binding column must be free of ethanol before RNA elution. Thus, in order to further dry the column, it was centrifuged for an additional 1 minute at maximum speed. Finally, the binding

column was transferred into another fresh 2 ml collection tube. 50 μL of the Elution Solution was added into the column and centrifuged at maximum speed for 1 minute. Purified RNA was now in the flow-through elute and ready for removal of DNA or storage at a -70℃ freezer.

2.2.4.2 Removal of DNA from RNA Extraction Samples

Total RNA samples were treated with DNase I to remove all possible DNA contaminations. Samples were firstly prepared as below:

- Total RNA (10-50μg): 50μl
- 10X incubation buffer: 6μl
- DNaseI, RNase free: 1μl
- DEPC water: 3μl

Then prepared samples were incubated at 37℃ for 20 minutes. In order to remove all DNase and salt, samples were passed again through the column as described previously. Purified DNA-free RNA samples were quantified by the measurement of A260/A280 absorbance.

2.2.4.3 Reverse Transcription-PCR (RT-PCR)

Reverse transcription-PCR (RT-PCR) is a PCR-based biological technique that converts an RNA chain to a complementary DNA sequence. This complementary DNA chain then further serves as a template for later DNA replication. For first strand cDNA synthesis, primed RNA was prepared as 2μg total RNA+1μl (0.5μg) oligo dT 18+DEPC water till total 10μl for 5 minutes 70℃ incubation. Meanwhile, the reaction mix was prepared as described below:

- 4μl 5X RT buffer
- 1μl RNase inhibitor
- 1μl 10mM dNTP mix
- 0.5μl (200U/μl) Reverse transcriptase BIOLINE
- Till 10μl diethylpyrocarbonate (DEPC) water

10μl reaction mix was added into incubated primed RNA for total 20μl volume and incubated at 42℃ for 1 hour. Finally, the reaction was stopped at 70℃ for 10 minutes and stored at -80℃.

2.2.4.4 qPCR

In this study, qPCR reaction was performed by Thermo Scientific Solaris qPCR Assay Kit. cDNA pool prepared from the previous reaction was diluted by

adding 30-40μl DEPC water. 1μl diluted cDNA pool was enough for each qPCR reaction.

**qPCR reaction**

Master Mix:

2X SensiMix SYBR: 5μl

10μM forward primer: 0.25μl (250nM final)

10μM reverse primer: 0.25μl (250nM final)

Water: up to 4.5μl

Template: 1μl diluted cDNA

Final volume per reaction: 10μl

qPCR Thermal Cycling Programme

| | Temperature | Time | Number of Cycles |
|---|---|---|---|
| Enzyme activation | 95℃ | 15 minutes | 1 cycle |
| Denaturation | 95℃ | 15 seconds | 40 cycles |
| Annealing/Extention | 60℃ | 60 seconds | |

CHEK1 Forward primer: ACTTCCGGCTTTCTAAGGGTGA

Reverse primer: GCAGGAAGCCAAATCTTCTG

Probe:ATTGATATTGTGAGCAGCC

ACTB Forward primer: TGGAGAAAATCTGGCACCAC

Reverse primer: GGTCTCAAACATGATCTGG

Probe: ACCGCGAGAAGATGACC

Data were analysed by SDS 2.2.1 programme.

### 2.2.5 Statistics

SPSS 10.0 (one way ANOVA followed by Post-hoc analysis with Bonferroni correction; SPSS Inc., Chicago, IL, USA) was applied for statistical analysis in this study. All the experiment results in this thesis were repeated at least three times. Values are means ± S.E. from at least 3 different samples. $P < 0.05$ was recognised the statistical significance between categories.

# CHAPTER 3 The response of normal urothelial cells to CHK1 inhibitors

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# Chapter 3 The response of normal urothelial cells to CHK1 inhibitors.

## 3.1 Introduction

It has been demonstrated that loss of cell cycle control enhances the accumulation of genetic alterations leading to transformation of normal cells to tumour cells, which eventually generates and develops to invasive, metastatic cancers. In response to DNA damage or replication stress, checkpoints are triggered to arrest cell cycle progression, which provides time for DNA repair. Activated checkpoints not only induce cell cycle arrest or cell death in response to some types of DNA damage (Nakano and Vousden, 2001; Yu et al., 2001) but also shown to protect tumour cells from apoptosis (Meuth, 2010; Rodriguez and Meuth, 2006; Sidi et al., 2008). In eukaryotic cells there are two highly conserved signalling cascades: two members of the phosphatidylinositol 3-kinase-like family of kinases, Ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) (Ciccia and Elledge, 2010) participate in these systems. Under DNA damage or replication stress, these activated kinases phosphorylate a number of downstream proteins to protect DNA integrity, including two key protein kinases, checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2) (Bartek and Lukas, 2003). Both CHK1/CHK2 are

serine/threonine protein kinases that participate in checkpoint signalling cascades, however they have been demonstrated to be activated by many different upstream regulators (Bartek and Lukas, 2003; Stracker et al., 2009). In general, the ATM-CHK2 signal transduction pathway responds to DNA double strand breaks (DSBs) or ends, however the ATR-CHK1 pathway is mainly activated in response to DNA replication stress and the accumulation of single stranded DNA at stalled replication forks. CHK1 is an unstable protein. Its expression is mainly restricted to S and $G_2$ phases (Kaneko et al., 1999). In contrast CHK2 is stable and expressed throughout the cell cycle. This feature honestly reflects the role of the ATM-CHK2 signalling cascade that is responsible for repairing DNA damages which may occur throughout the cell cycle (Lukas et al., 2001).

Recently the interest in the use of CHK1 inhibitors in cancer therapy has grown dramatically (Bennett et al., 2012; Blackwood et al., 2013; Carrassa and Damia, 2011; Cole et al., 2011). CHK1 kinase has been reported not only to regulate cell cycle progression from S to M phase following disruption of DNA replication or some types of DNA lesion but also play a role in S phase procession even in undisturbed cells (Petermann and Helleday, 2010;

Petermann et al., 2006). CHK1 plays a vital role in mammalian development and viability. Once CHK1 is knocked out, it causes embryonic lethality in mice (Liu et al., 2000; Takai et al., 2000). Cells cultured from early embryos of such strains have exhibited failure to survive and shown extensive cell apoptosis (Takai et al., 2000). Although CHK1 knockdown or depletion is not invariably lethal, delayed cell growth with a particularly slower progression through S-phase and increased levels of spontaneous apoptosis have been shown in many cell types depleted of CHK1 (Petermann et al., 2006; Zachos et al., 2003). However when DNA replication is disrupted, siRNA-mediated CHK1 depletion or small molecule inhibition of CHK1 have been demonstrated to trigger cell death in many cell types (Myers et al., 2009; Rodriguez and Meuth, 2006). These findings indicate that CHK1 plays a role in protecting cells from apoptosis caused by DNA replication stress. However, the principal cell models utilized for CHK1 studies have been either cancer cell lines or immortalized fibroblasts. Due to the fact that most tumours have an epithelial origin, it is worthwhile to understand the role of these pathways in normal epithelial cells for more effectively target therapy developments to beat developing tumour cells.

## 3.2 Aims and Hypothesis

While DNA damage response pathways are well characterized in cancer cells, much less is known about their status in normal cells. These pathways protect tumour cells from endogenous DNA damage and oncogene-induced replication stress and consequently present potential therapeutic targets. Given that normal cells may be less prone to such stresses, the hypothesis of this project was that the DNA damage response may be very different in normal human epithelial cells. To test this hypothesis, the responses of both primary and hTERT-immortalized normal human urothelial (NHU) cells to DNA replication inhibitors were characterised. In this work, the effects of replication inhibitors on cell cycle progression, checkpoint induction, and apoptosis were analysed by FACS, Western blotting and immunofluorescence in these cells.

## 3.3 Results

### 3.3.1 CHK1 inhibition slows S-phase progression in response to DNA replication stress in both primary and hTERT-NHU cells but does not trigger apoptosis.

To determine the response of non-malignant epithelial cells to DNA replication stress, hTERT-NHU cells were treated with thymidine for 24 or 48hr in the presence or absence of the CHK1 inhibitor Gö6976. Cells were harvested and analysed for DNA content by flow cytometry. Unlike the malignant tumour cells which were previously reported in 2010 (Gagou et al., 2010), these non-malignant urothelial cells showed totally different response. Cells treated with the CHK1 inhibitor alone for 24 or 48hr did not show any significant alteration in cell cycle distribution compared with untreated control (Fig. 3.1). Cultures treated with thymidine showed a significant accumulation of cells in S-phase, which stand for a slow progression through S-phase. Following 24hr treatment with a combination of thymidine and Gö6976, cells still arrested in S-phase, which is similar to those treated with thymidine alone (Fig. 3.1A). However when exposure time was extended to 48hr, hTERT-NHU cells under the combination treatment failed to progress further through S-phase while the cells treated with thymidine alone were able to progress slowly through S and

accumulated in $G_2$ (Fig. 3.1B). Notably, only few hTERT-NHU cells treated with the combination showed a sub-$G_1$ population which is characterised as apoptotic cells.

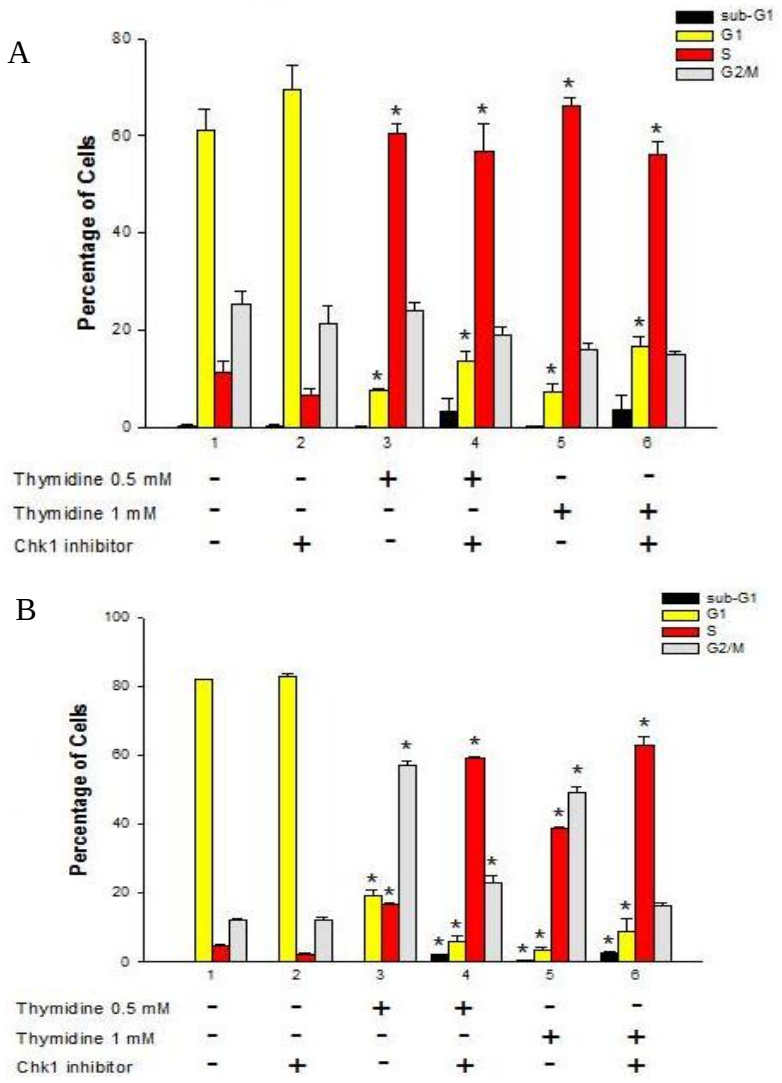



**FIGURE3. 1. CHK1 inhibitor slows S-phase progression in hTERT-NHU cells in response to replication stress but does not trigger death.**
hTERT-NHU cells were pre-treated with 1μM CHK1 inhibitor (Gö6976) for 2h and then incubated with or without 1mM thymidine for A) 24h and B) 48h before harvest and analysis for cell cycle distribution by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. *P<0.05 compared with untreated control cells. Sub$G_1$ population of EJ cells serves as positive control (presented as Fig. 4.1A, Chapter 4)

To further examine the effect of Chk1 inhibition in normal urothelial cells upon DNA replication stress, a second Chk1 inhibitor, CHIR-124 was also tested in hTERT-NHU cells in response to 24hr thymidine treatment. In contrast to Gö6976, Chir124 arrested hTERT-NHU cells in late S/G2/M but still failed to trigger cell death (Fig. 3.1C).

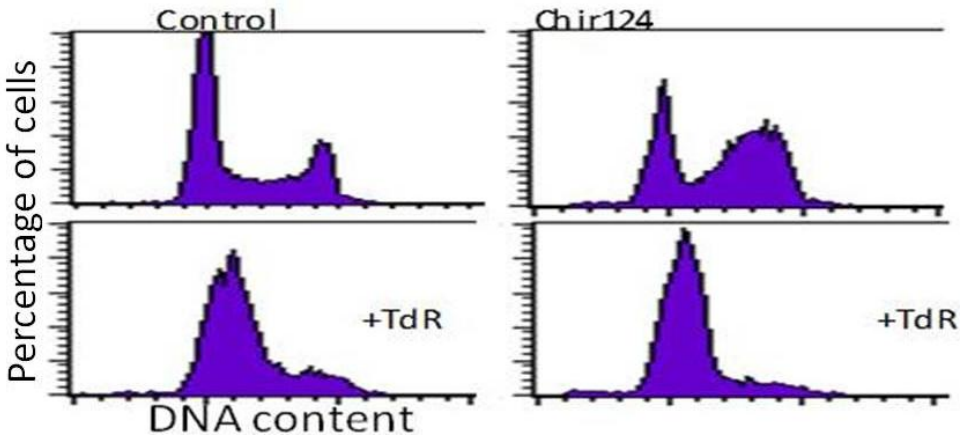



**FIGURE3.1C. Other CHK1 inhibitor also fail to induce apoptosis in hTERT-NHU cells.** Representative profiles of hTERT-NHU cells treated with the CHK1 inhibitor Chir124 (right panels) in the presence or absence of thymidine (lower panels).

Furthermore, treated primary human urothelial cells were similarly treated to see their responses to Chk1 inhibitor and/or DNA replication stress. Like the hTERT-immortalized cells, these cells accumulated in S- and $G_2$/M phase in response to thymidine or thymidine and Gö6976, but no significant sub-$G_1$ population was detected (Fig. 3.1D).

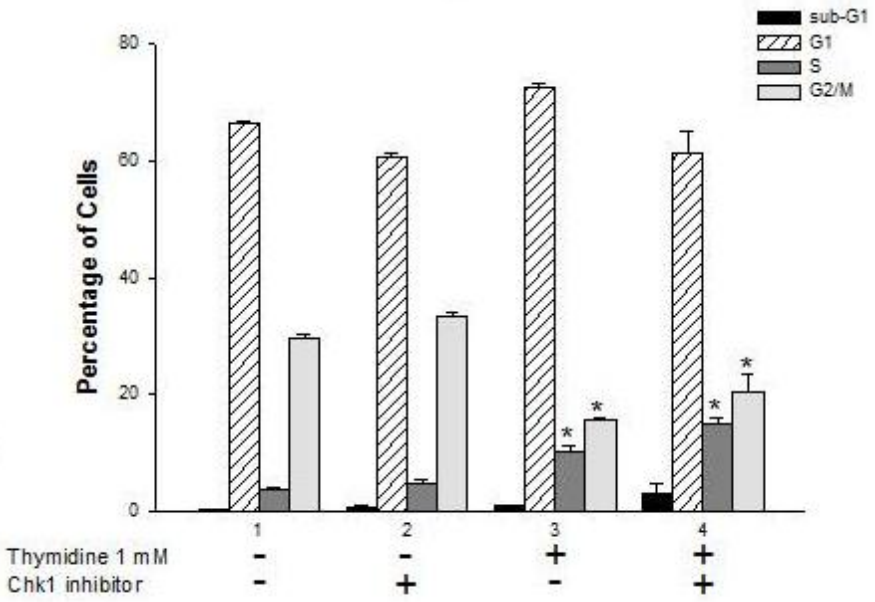



**FIGURE 3.1D. CHK1 inhibitor slows S-phase progression in primary NHU cells in response to replication stress but does not trigger death.**
Primary NHU cells were pre-treated with 1μM CHK1 inhibitor (Gö6976) for 2h and then incubated with or without 1mM thymidine for 24 before harvest and analysis for cell cycle distribution by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. *P<0.05 compared with untreated control cells.

Additionally, hTERT-NHU cells treated with another replication inhibitor, hydroxyurea in the presence or absence of the Gö6976 were also analysed. Like the treatment of thymidine, hydroxyurea only triggered S-phase accumulation without significant induction of sub-$G_1$ population (Fig. 3.1E).

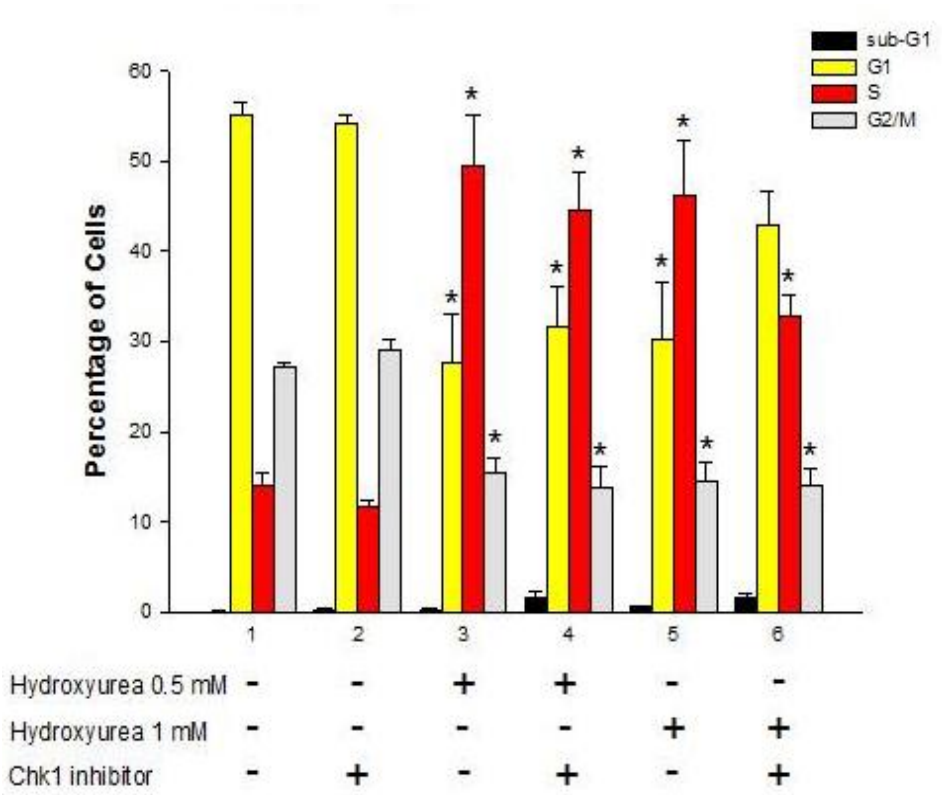



**FIGURE 3.1E. Other replication inhibitors also fail to induce apoptosis in hTERT-NHU cells.** hTERT-NHU cells were pre-treated with 1 mM CHK1 inhibitor (Gö6976, 1μM, right panels) for 2 hr before addition of 0.5 or 1 mM HU for 24h (lower panels). Cells were then harvested and analysed for DNA content. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

### 3.3.2 The role of Chk1 in hTERT-NHU cells in response to clinical anti-cancer drug gemcitabine.

The effect of Gö6976 on the response to the deoxycytidine analog gemcitabine which is widely used in the treatment of advanced bladder cancer was further examined. Cells were treated with 12.5 nM gemcitabine in the presence or absence of a 2hr pre-incubation with Gö6976. Consistent with the response triggered by thymidine, no significant cell death was induced by a 24h co-treatment with gemcitabine and Gö6976 in hTERT-NHU cells (Fig. 3.2).

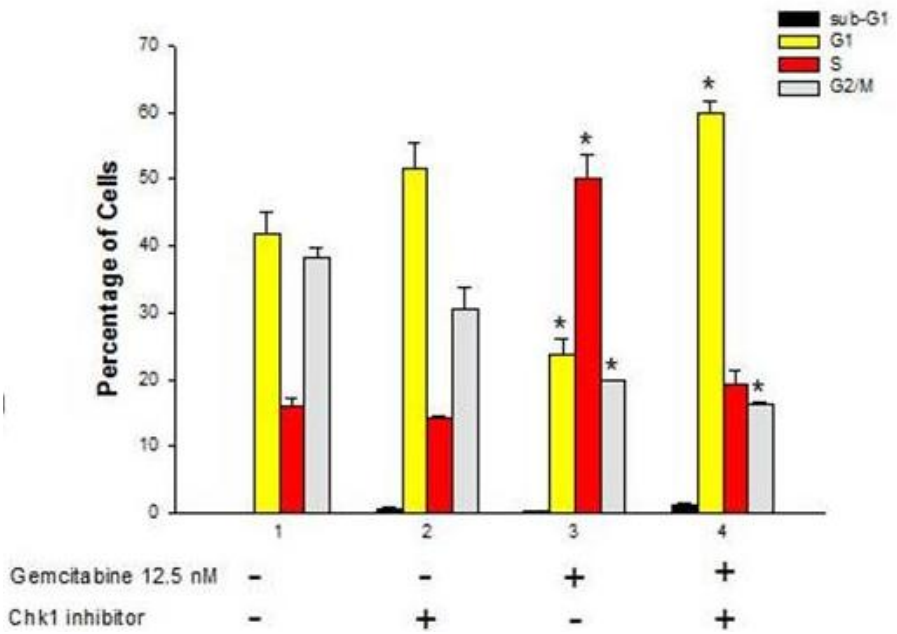



**FIGURE3. 2A. Gemcitabine induces no apoptosis in hTERT-NHU cells when CHK1 is inhibited.** hTERT-NHU cells were pre-treated with 1 M Gö6976 for 2 h and then incubated with or without 12.5nM Gemcitabine for 24h before harvest and analysis for cell cycle distribution by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. * $P<0.05$ compared with untreated negative control (NC) cells. Note: The induction of sub-$G_1$ populations in EJ cells serves as a positive control (presented as Fig. 4.3)

Taken together, these results show that the combination of DNA replication stress agent and small molecule-caused CHK1 inhibition is unable to trigger cell death in both primary and hTERT-immortalized NHU cells.

### 3.3.3 Western blot analysis of DNA damage response proteins in Chk1 inhibited hTERT-NHU cells treated with thymidine.

To determine how regulatory CHK1 participates in checkpoint mechanism and cell cycle regulation in hTERT-NHU cells exposed to replication inhibitors, cultures were treated with thymidine following the presence or absence of Gö6976 treatment for different time points and harvested for Western blot analysis as well. CHK1 autophosphorylation at Ser296 readily appeared in both untreated control and thymidine treated hTERT-NHU cells. This event was completely suppressed by Gö6976, demonstrating the effectiveness of the kinase inhibition. In contrast, the crucial Ser345 phosphorylation of CHK1 could not be detected even after a 24hr thymidine treatment while CHK1 pSer317 phosphorylation was transiently observed after an 8hr treatment (Fig. 3.3A).

In contrast, although ATM showed an early response (presented in Chapter 5) Chk2 seems only poorly affected in this signalling transduction cascade as only little phosphorylated and total protein level of Chk2 showed apparent changes in hTERT-NHU cells in response to Gö6976-induced CHK1 inhibition (Fig. 3.3A).

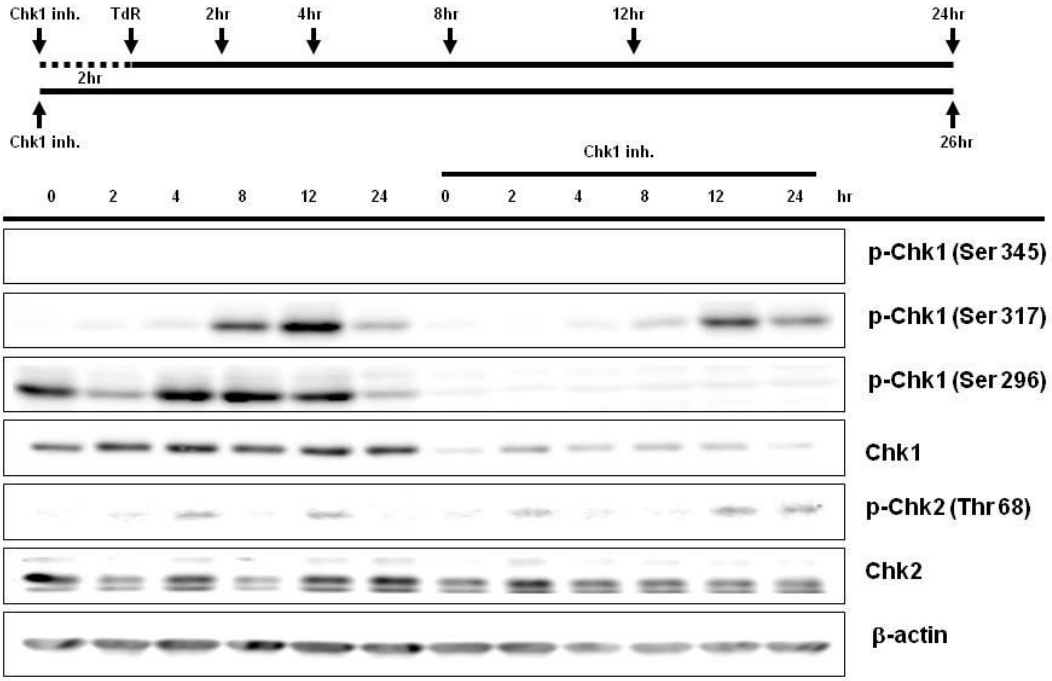



**FIGURE3. 3A. Western blot analysis of Chk1/2 phosphorylation in hTERT-NHU cells treated with thymidine in the presence or absence of CHK1 inhibitor.** hTERT-NHU cells were exposed to 1mM thymidine for 0-24h in the presence or absence of 1μM Gö6976 before harvest and preparation of cell free extracts. Cells treated with Gö6976 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the CHK1 inhibitor alone (0h thymidine) were exposed for 26h. Western blots prepared from these extracts were analysed for the indicated phosphorylated Chkl and Chk2 proteins. β-actin levels are presented as loading controls. Note: p-Chk2 (Thr68) expression was weak, thus the exposure time for image capture system was more than 2hr; Expression of p-Chk1 Ser345 in EJ cells serves as a positive control (presented as Fig. 4.2A, Chapter 4)

Total levels of other damage signaling response associated proteins in hTERT-NHU cells were also examined in response to the treatment. p53 was stabilized and phosphorylated after 12hr treatment while the total protein level of CHK1 strikingly decreased in cells following CHK1 inhibitor treated in either presence or absence of thymidine (Fig. 3.3B). Notably, while pSer139 H2AX

(γ-H2AX) was only weakly detected in hTERT-NHU cells treated with thymidine alone, both RPA and H2AX phosphorylation were apparently detected at later times in cells following the combined treatment of Gö6976 and thymidine (Fig. 3.3B).

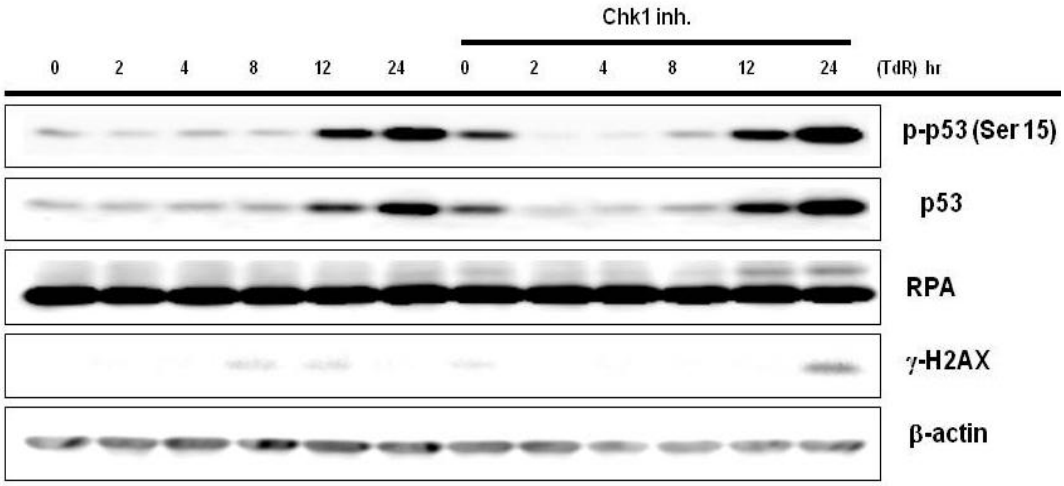
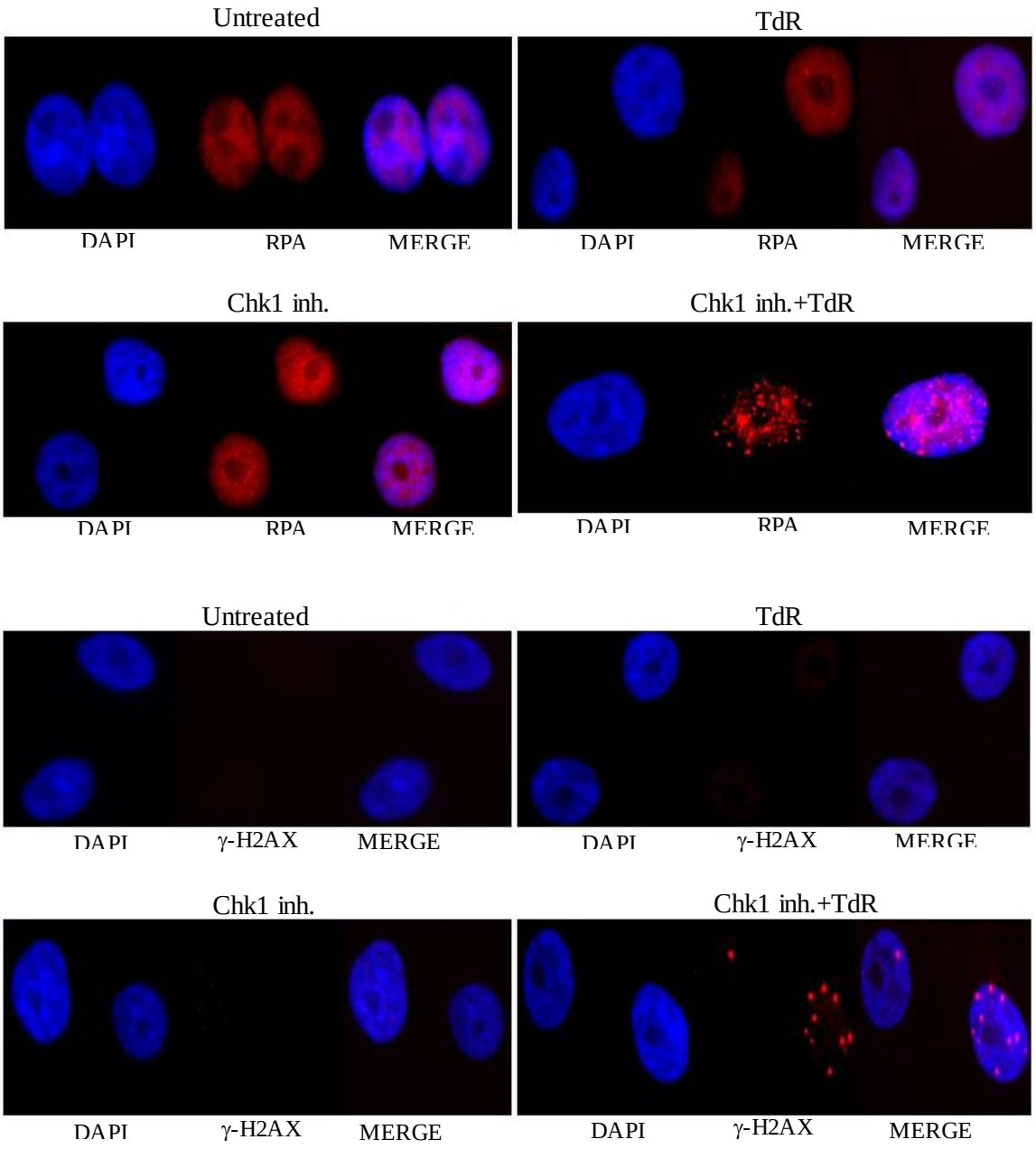



**FIGURE 3.3B. Western blot analysis of DNA damage response proteins in hTERT-NHU cells treated with thymidine in the presence or absence of CHK1 inhibitor.** hTERT-NHU cells were exposed to 1mM thymidine for 0-24h in the presence or absence of 1μM Gö6976 before harvest and preparation of cell free extracts. Cells treated with Gö6976 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the CHK1 inhibitor alone (0h thymidine) were exposed for 24h. Western blots prepared from these extracts were analysed for the indicated damage response proteins. β-actin levels are presented as loading controls.

### 3.3.4 RPA and γ-H2AX foci are activated in hTERT-NHU in response to the combination of CHK1 and replication inhibitors.

The foci formation by RPA and γ-H2AX are further confirmed by immunofluorescence. Combined treatment of CHK1 inhibitor and thymidine showed enhanced RPA and γ-H2AX foci formation in hTERT-NHU cells relative to cells treated with thymidine or Gö6976 alone (Fig. 3.4). However, compared with the previous report based on tumour cells such as HCT116, the γ-H2AX foci were more distinct and localized in hTERT-NHU cells and the pan-nuclear γ-H2AX staining found in tumour cells caused by these conditions (Gagou et al., 2010) was not evident in hTERT-NHU cells either.


Untreated
TdR
DAPI
RPA
MERGE
DAPI
RPA
MERGE
Chk1 inh.
Chk1 inh.+TdR
DAPI
RPA
MERGE
DAPI
RPA
MERGE
Untreated
TdR
DAPI
γ-H2AX
MERGE
DAPI
γ-H2AX
MERGE
Chk1 inh.
Chk1 inh.+TdR
DAPI
γ-H2AX
MERGE
DAPI
γ-H2AX
MERGE

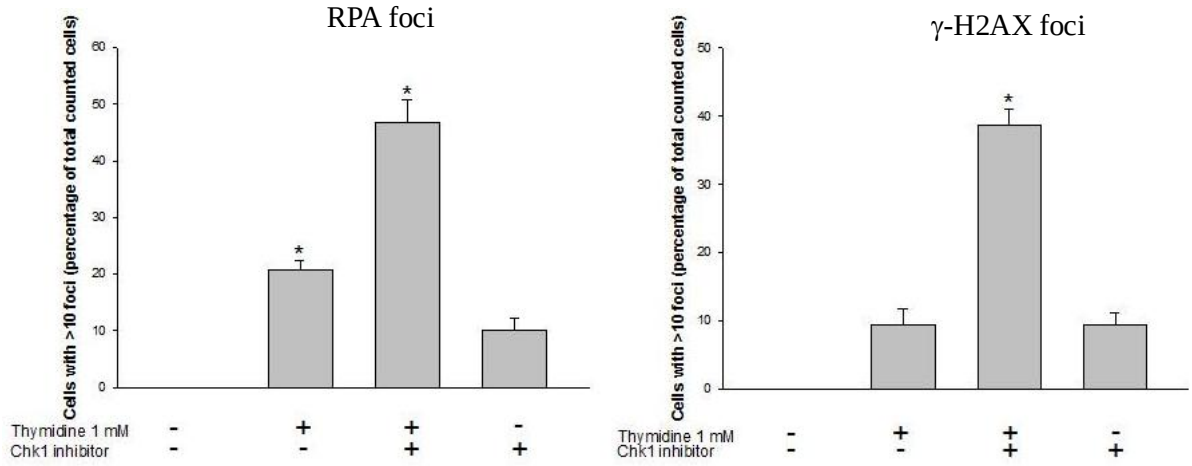



**FIGURE3. 4. Immunofluorescence analysis of RPA and γ-H2AX foci in hTERT-NHU cells treated with thymidine in the presence or absence of CHK1 inhibitor.** Representative images from immunofluorescence analysis of RPA and γ-H2AX foci in hTERT-NHU cells treated with 1mM thymidine and 1μM Gö6976. Due to the multiple phosphorylation sites of RPA, the antibody against total RPA was used in these experiments. Bar graphs present the mean of at least three independent experiments ±standard deviation of hTERT-NHU cells treated with 1mM thymidine for 24h with or without Gö6976 (1μM). *P<0.05 compared with untreated control cells.

### 3.3.5 RPA is only partially phosphorylated in hTERT-NHU in response to DNA replication inhibition.

To further confirm whether replication stress-induced RPA hyperphosphorylation in CHK1 inhibited hTERT-NHU cells is associated with ATR-Chk1 pathway, different phosphorylation sites of RPA was examined by immunoblotting. Interestingly, RPA was only phosphorylated at Ser4/Ser8 but no Ser33 phosphorylation was found in these cells (Fig. 3.5). Notably, compared with chemical inhibition of the kinase inhibitor, HCT116 was considered as a positive control with its siRNA-caused cellular protein depletion.

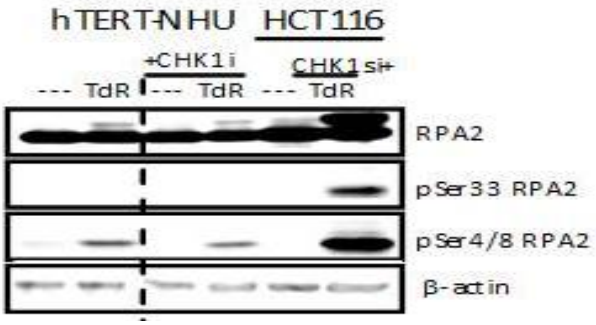



**FIGURE3. 5. Western blot analysis of phosphorylated forms of RPA2 in extracts prepared from hTERT-NHU cells.** hTERT-NHU cells treated with 1mM thymidine for 24h with or without Gö6976 (1μM). HCT116 cells treated with CHK1 siRNA and thymidine were generously gifted by Dr. Zuazua-Villar and presented as a positive control for full hyperphosphorylation of RPA2 (Gagou et al., 2010; Rodriguez et al., 2008).

Since it has been shown that Ser33 is phosphorylated by ATR under such conditions (Anantha et al., 2007; Vassin et al., 2009), the absence of Ser33

phosphorylation further suggests that ATR-CHK1 signalling may not be fully activated in response to CHK1 and replication inhibitors in the hTERT-NHU cells. This suggestion was further confirmed by the evidence of reduced RAD51 foci in response to DNA replication stress. CHK1-dependent RAD51 has been known to form foci at stalled forks during DNA replication arrest (Sorensen et al., 2005). Compared with HCT116 colon cancer cells which are known to form RAD51 foci in response to replication stress, only 25% of hTERT-NHU cells showed >10 RAD51 foci following thymidine treatment whereas more than 90% of HCT116 cells presented a similar response (Fig. 3.6).

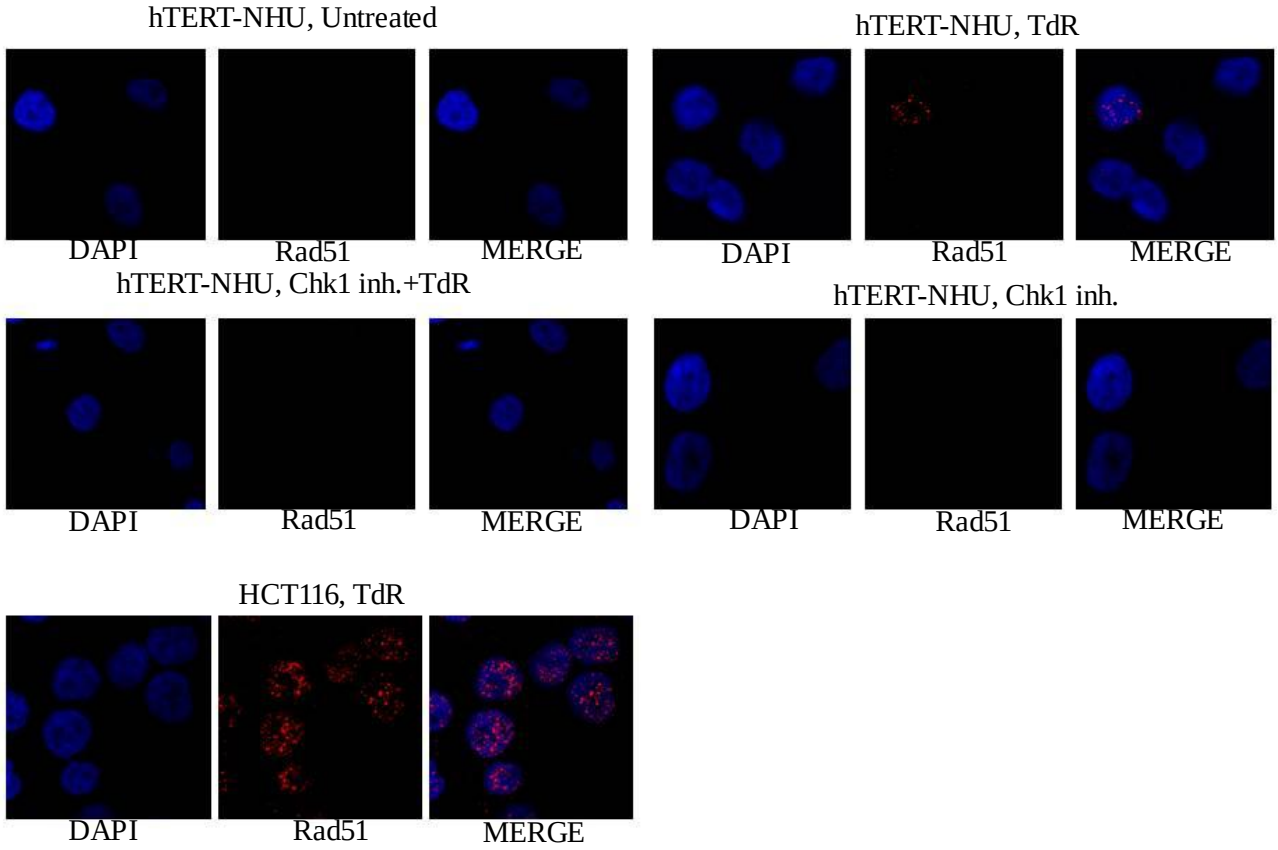
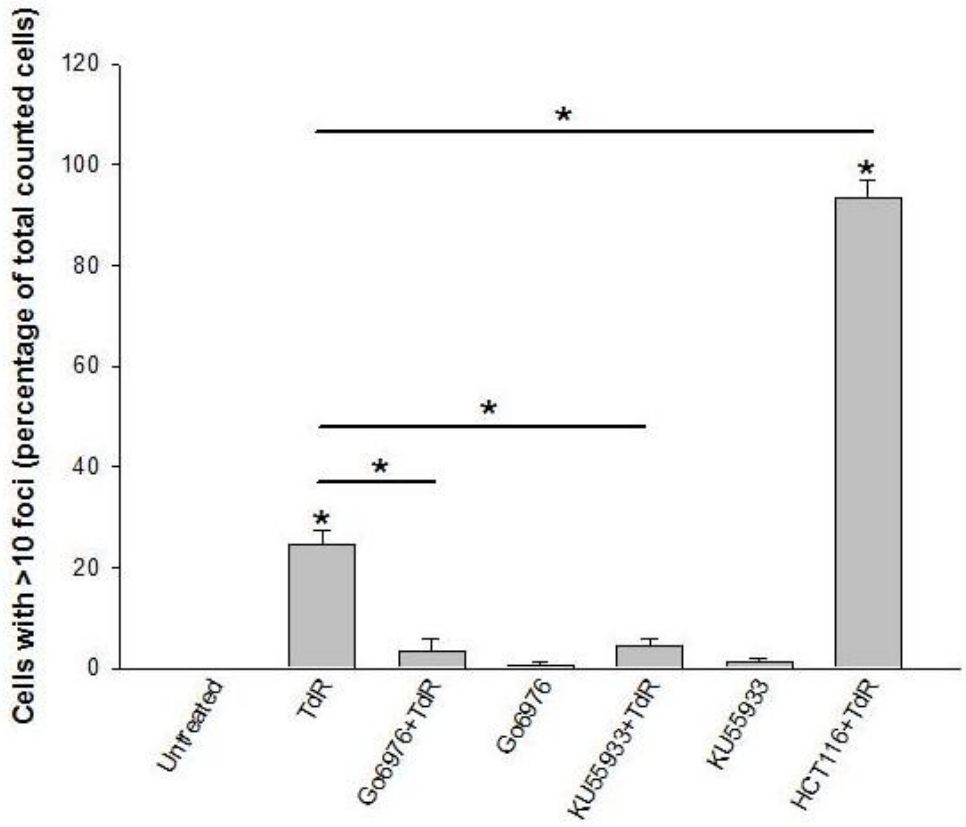


**FIGURE3. 6. Suppression of RAD51 foci formation by CHK1 inhibition in hTERT-NHU cells following treatment with thymidine.** hTERT-NHU or HCT116 cells treated with 1mM thymidine in the presence or absence of the CHK1 inhibitor Gö6976 (1μM) for 24h were fixed and stained for DNA content and RAD51. Images from immunofluorescence analysis represent RAD51 foci in hTERT-NHU or HCT116 cells treated with thymidine. The percentages of cells showing >10 RAD51 foci were scored.




Bar graphs present the mean of at least three independent experiments ±standard deviation of hTERT-NHU cells or HCT116 cells.

In addition, the foci formed in the hTERT-NHU cells were less intense than those arising in HCT116 and they were sensitive to Gö6976, which indicates that even not fully activated ATR-CHK1 in some cells was able to induce formation of RAD51 foci.

Taken together, these data suggest that there is a lack of activation of the ATR-CHK1 signalling cascade in hTERT-NHU cells in response to DNA replication stress.

### 3.3.6 Reduction of CHK1 protein levels is accompanied by lower transcript levels.

As described previously, CHK1 protein level was found to decrease in response to Gö6976 (Fig. 3.3A). As Gö6976 does not degrade protein level directly but only inhibit the CHK1 Ser296 autophosphorylation in response to DNA replication stress, the data has revealed a new question that where have the proteins gone? Recently, CHK1 was found to be ubiquitinated by the Skp1-Cul1-Fbx6 E3 ligase for proteasome-mediated degradation following phosphorylation of CHK1 at Ser345 in response to DNA replication stress (Zhang et al., 2009). Although there was no phosphorylation of CHK1 Ser345 detected in hTERT-NHU cells in response to thymidine treatment, whether the lower total level of CHK1 in such cells was the result of proteasome associated degradation of CHK1 was still examined. hTERT-NHU cells were pre-treated with the proteosome inhibitor MG-132 followed by a exposure of Gö6976 and thymidine. Unlike the response of tumour cells (Wang et al., 2005), the lower CHK1 levels caused by Gö6976 treatment were not restored by treatment with MG-132 (Fig. 3.7A). Interestingly, qPCR analysis showed that Gö6976 treated hTERT-NHU cells had a more than 2-fold lower RNA level compared with untreated control (Fig. 3.7B). Thus the decrease CHK1 protein level detected

in response to treatment with the Gö6976 CHK1 kinase inhibitor appears to be the result of decreased transcription.


**FIGURE3. 7. Decreased CHK1 transcription in hTERT-NHU cells treated with CHK1 inhibitor.** **(**A) Western blot analysis of hTERT-NHU cells treated with Gö6976 (1μM) for the indicated times in the presence or absence of the proteasome inhibitor MG132. pSer296 CHK1 is presented to confirm CHK1 inhibition and β-actin as loading control. (B) qPCR analysis of CHK1 transcripts in hTERT-NHU cells grown in the presence or absence of Gö6976 (1μM) for 24h.

## 3.4 Discussion

It has been well agreed that DNA damage response proteins regulate cell cycle progression in response to DNA damage or replication disruption. Many of these regulators are also important for optimal growth even in undisturbed cells. There have been many works from different laboratories shown that CHK1 not only regulates S-phase progression in cancer cells but also protects them from apoptosis in response to DNA replication stress (Meuth, 2010; Petermann et al., 2006; Rodriguez and Meuth, 2006; Sidi et al., 2008). However, the role of these checkpoint response proteins in “normal” epithelial cells is less clear. Here I investigated the response of primary and hTERT-immortalized NHU cells to variant DNA replication stresses. In contrast to many other reported studies with cancer cell lines, CHK1 inhibitors trigger very little cell death in response to DNA replication stress in both primary and hTERT-immortalized NHU cells. This indicates that although CHK1 is required for efficient progression of normal human urothelial cells through S-phase during DNA replication stress but it is not necessary to protect such cells from apoptosis.

In contrast to the response reported by other studies based on cancer cell lines, the data also suggest that CHK1 is only partially activated in hTERT-NHU cells following treated with a replication inhibitor. CHK1 is largely activated in response to ssDNA formation due to occurrence of functional uncoupled DNA polymerase and MCM helicase following replication inhibition (Byun et al., 2005). This ssDNA is rapidly coated by RPA which then recruits ATR through the ATR interacting protein (ATRIP) (Zou and Elledge, 2003). The formation of this complex activates downstream CHK1 through phosphorylation at Ser345 and Ser317 (Green et al., 2000; Zhao and Piwnica-Worms, 2001). Interestingly, this critical phosphorylation of CHK1 (on Ser345) in response to thymidine treatment is not found in hTERT-NHU cells. CHK1 S345A mutations result in impaired cell cycle checkpoints, loss of viability for proper localization and lead to apoptosis due to mitotic catastrophe in many types of cells(Shimada et al., 2008; Walker et al., 2009; Wilsker et al., 2008). Thus since Chk1 S345 is essential for all functions of its kinase activity, the failure to phosphorylate this crucial site would be likely to severely reduce the CHK1-mediated checkpoint response.

The data suggest that this lack of Ser345 phosphorylation is because of the failure of hTERT-NHU cells to activate ATR in response to replication stress. It has previously been shown that RPA2 has a distinctive pattern of phosphorylation in many tumour cell lines in response to replication and CHK1 inhibitors (Anantha et al., 2007; Liu et al., 2012). There is general agreement that Ser33 is a phosphorylation target for activated ATR (Anantha et al., 2007; Vassin et al., 2009). In contrast, Ser4/8 is mainly phosphorylated through ATM or DNAPK activity (Liu et al., 2012). Ser4/8 phosphorylation of RPA is usually found only in the most hyper-phosphorylated forms of RPA2 during DNA replication stress following phosphorylation of Ser33 which is phosphorylated by ATR (Anantha et al., 2007).

Furthermore, when DNA replication is arrested, the stalled forks recruit a number of DNA repair associated proteins such as the homologous recombination repair protein RAD51 to resolve damage lesions and restart replication. RAD51 forms foci at stalled forks during DNA replication stress and this response has been strongly demonstrated as CHK1-dependent (Sorensen et al., 2005). Thus the lack of Ser33 phosphorylation and weak Rad51 foci in hTERT-NHU cells treated with thymidine in the presence or absence of CHK1

inhibitors combined with the Ser4/8 phosphorylation suggests that the ATR-CHK1 pathway is not fully activated in these cells and that ATM or DNAPK signalling takes the duty of the initial sensor in response to replication fork stress in these immortalized epithelial cells.

Since Ser345 phosphorylation of CHK1 is required for the F box protein (Fbx6)-mediated degradation of CHK1 (Zhang et al., 2009), the absence of this phosphorylation in hTERT-NHU cells is likely to strengthen the stability of CHK1 during thymidine treatment. In contrast, treatment with Gö6976 down regulated CHK1 total protein level in either presence or absence of thymidine and a proteasome inhibitor failed to restore CHK1 levels. qPCR analysis indicated that the decrease of CHK1 is due to lower levels of CHK1 transcription. This strongly suggests that CHK1 kinase activity may be required to maintain its own protein level in hTERT-NHU cells even in undisturbed cells. CHK1 has also previously been reported to dissociate from chromatin in response to DNA damage (Smits et al., 2006) and correlate with rapidly reduced phosphorylation of histone H3 on residue Thr11 (Klionsky et al., 2008). This has been shown to be necessary for facilitating the transmission of DNA-damage signals to downstream targets and repressing the transcription

of some regulators of cell cycle progression (Klionsky et al., 2008; Smits et al., 2006), although the previous study did not reveal whether the CHK1 kinase regulated its own level.

# Chapter 4 Activation of the Chk1 suppressed death in advanced malignant tumour cells under DNA replication stress

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# Chapter 4 Activation of the Chk1 suppressed death in advanced malignant tumour cells under DNA replication stress

## 4.1 Introduction

The role of Chk1 in the cellular response to DNA replication stress is well examined. In addition to the regulation of cell cycle progression and checkpoint mechanism, recent studies reported a novel role of Chk1 in the suppression of apoptosis resulted from the disruption of DNA replication or DNA damage in variant tumour cell types (Cho et al., 2005; Rodriguez and Meuth, 2006; Sidi et al., 2008). ATR and Chk1 were showed to suppress apoptotic response by a caspase 3-dependent manner following DNA replication stress in both p53-proficient and -deficient cells (Myers et al., 2009). However, since Chk1 has a number of roles in cells in response to DNA replication stress, the precise molecular mechanisms of how loss of CHK1 triggers apoptosis following replication disruption is still not clear.

## 4.2 Aims and Hypothesis

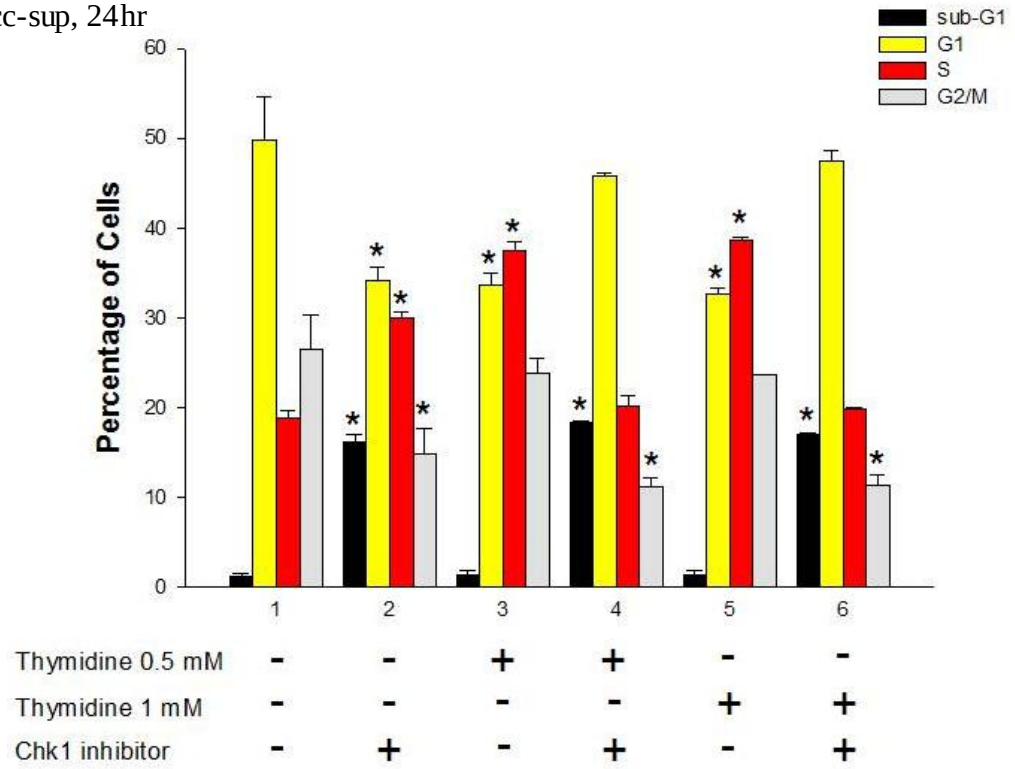
The role of Chk1 has been well characterised in various studies that Chk1 not only participates in cell cycle progression in undisturbed cells, but also in checkpoint system and protect tumour cells from cell death in response to DNA replication defects. This indicates a novel road toward cancer therapy. Thus, it is hypothesised that bladder tumour cells also show the reliance of cell survival on Chk1. In order to test the hypothesis and examine the potential role of CHK1 on targeted therapy in bladder cancers, various bladder cancer cell lines including two advanced malignant tumours Tcc-sup and EJ were investigated. In this present research, while cell cycle distribution and cell death were examined by flow cytometry, protein activation and total protein level changes were studied by immunoblotting.

## 4.3 Results

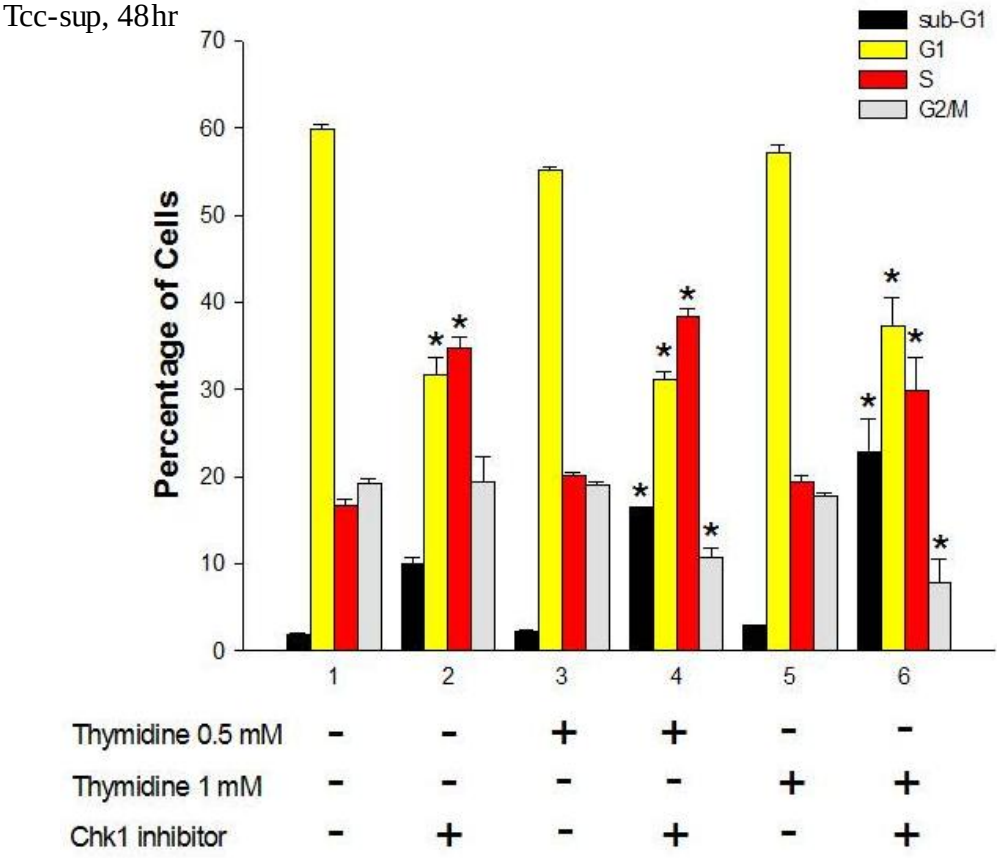
### 4.3.1 Thymidine-induced DNA replication stress leads to S phase arrest with apparent cell death in Chk1 inhibited advanced bladder cancer cells.

In the beginning, I tested the role of Chk1 in response to DNA replication stress in two different malignant bladder tumour cell lines. Consistent with other published reports that Chk1 plays a role in protecting cancer cells from cell death upon DNA replication defects (Gagou et al., 2010), treatment of bladder cancer cell lines (TCC-sup and EJ) with thymidine and Gö6976 produced very strong death effects of sub-$G_1$ DNA content accumulation. Treatment of TCC-sup cells with Gö6976 or thymidine slowed S-phase transition with highly increased duplicated DNA content while a 24 or 48h co-treatment with these two inhibitors dramatically boosted levels of cells with a sub-$G_1$ DNA content relative to cells treated with thymidine alone (Fig. 4.1A). Strikingly, even Gö6976 alone triggered a robust apoptotic response after only 24h with a high sub-$G_1$ peak, but no accumulation in S-phase in EJ cells. When exposure to the combination of the two agents, the level of cells with sub-$G_1$ DNA content was increased much further (Fig. 4.1A).

Tcc-sup, 24hr




Tcc-sup, 48hr



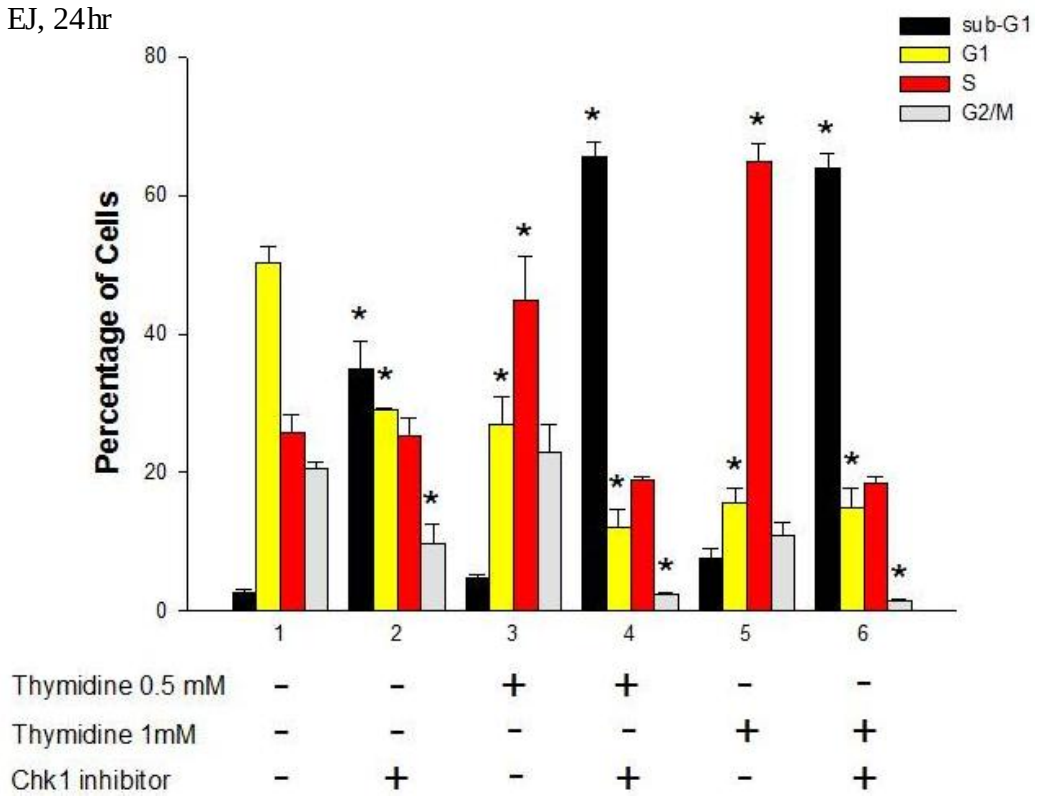



**FIGURE4.1A. Thymidine-induced DNA replication stress lead to S phase arrest with apparent cell death in Chk1 inhibited TCC-sup and EJ cells.** Tcc-sup and EJ were pre-treated with 1μM CHK1 inhibitor (Gö6976) for 2h and then incubated with or without 1mM thymidine for 24h or 48h before harvest and analysis for cell cycle distribution by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. *P<0.05 compared with untreated control cells.

To ensure the sub-$G_1$ DNA content was caused due to CHK1 inhibition-induced apoptosis, caspase3 the marker of apoptosis was examined in response to the treatment as well. Consistently, the increased level of the sub-$G_1$ population in EJ treated with Gö6976 was accompanied by a dramatic increase in the level of activated-caspase3 (Fig. 4.1B).

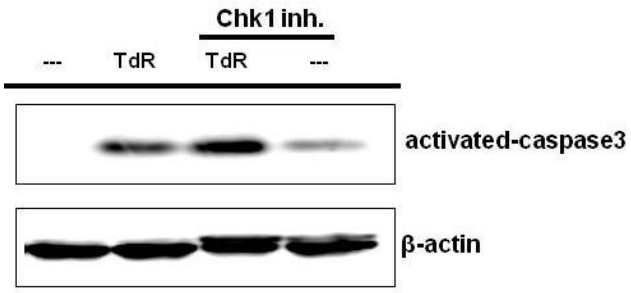



**FIGURE4.1B. Thymidine-induced DNA replication stress lead to apparent cell death in Chk1 inhibited EJ cells.** Western blot analysis of activated-caspase3 in hTERT-NHU or EJ cells treated with 1μM Gö6976 and/or 1mM thymidine. β-actin levels are presented as loading controls.

### 4.3.2 Thymidine-induced DNA replication stress leads to the classical ATR-Chk1 activation in EJ cells.

I further examined the protein activation status and total protein levels of EJ cells in response to these treatments. It is commonly accepted that while CHK1 is rapidly phosphorylated at Ser345 and Ser317 in response to DNA damage or replication stress in a wide range of cells, the autophosphorylation site at Ser296 within the c-terminal regulatory domain (Clarke and Clarke, 2005) is phosphorylated even in the absence of replication stress (Wilsker et al., 2008). Both Ser345 and Ser317 are critical for CHK1 kinase function as serine-to-alanine substitution mutations (S345A or S317A) that eliminate phosphorylation at these sites leading to suppress checkpoint activation and reduce viability (Shimada et al., 2008; Walker et al., 2009; Wilsker et al., 2008). In this case, cultures were exposed to thymidine in the presence or absence of Gö6976 pre-treatment for variant time points and harvested for Western blot analysis. Like the result obtained in normal human urothelial cell which was described in previous chapter, the elimination of Chk1 Ser296 expression in response to the treatment of CHK1 inhibitor confirmed the efficiency of the inhibitor. Consistent with other types of tumour cells, EJ bladder cancer cells show apparent phosphorylation of CHK1 at both Ser345 and Ser317 within 2h

of thymidine treatment. These events were further enhanced when tumour cells were co-treated with CHK1 inhibitor (Fig. 4.2A) whereas both phosphorylated- and total protein level of ATM were increased in response to DNA replication stress and not suppressed following Chk1 inhibition (presented in chapter 5).

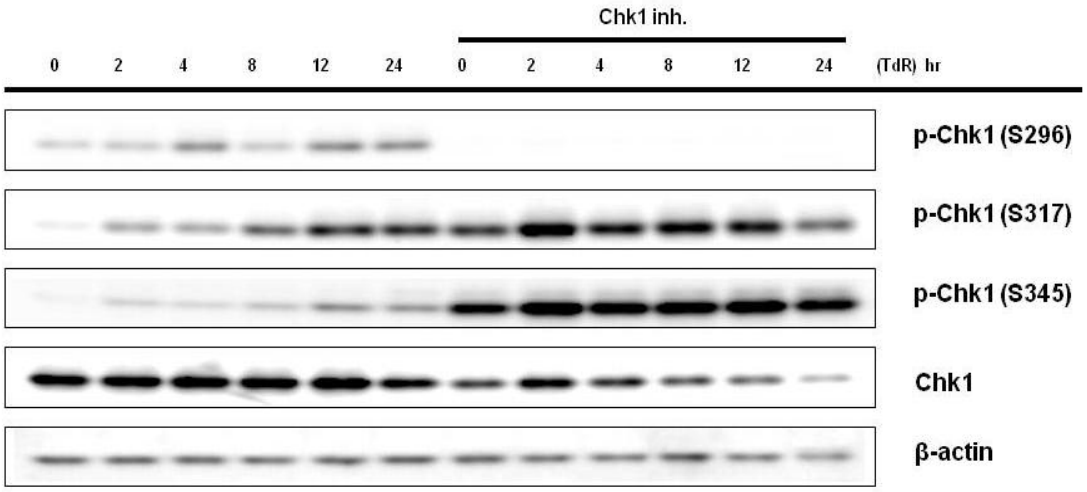



**FIGURE4.2A. Western blot analysis of Chk1 activation in EJ cells treated with thymidine in the presence or absence of CHK1 inhibitor.** EJ cells were exposed to 1mM thymidine for 0-24h in the presence or absence of 1μM Gö6976 before harvest and preparation of cell free extracts. Cells treated with Gö6976 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the CHK1 inhibitor alone (0h thymidine) were exposed for 24h. Western blots prepared from these extracts were analysed for Chk1. β-actin levels are presented as loading controls.

Furthermore, the Replication Protein A2 (RPA2) subunit of the RPA complex and histone H2AX were also reported to be phosphorylated in response to DNA replication disruption (Siegel et al., 2014; Syljuasen et al., 2005). The

data indicate that these events were further amplified in tumour cell lines treated with CHK1 inhibitors or siRNA (Fig. 4.2B). Although Chk2 Thr-68 and p53 Ser-15 phosphorylation were detected by immunoblotting, they do not seem to be the members participating in the checkpoint system that ATR-Chk1 signalling cascades also plays, in response to thymidine-induced replication defects as CHK1 inhibited by Gö6976 does not result in obvious changes on their phosphorylation (Fig. 4.2B).

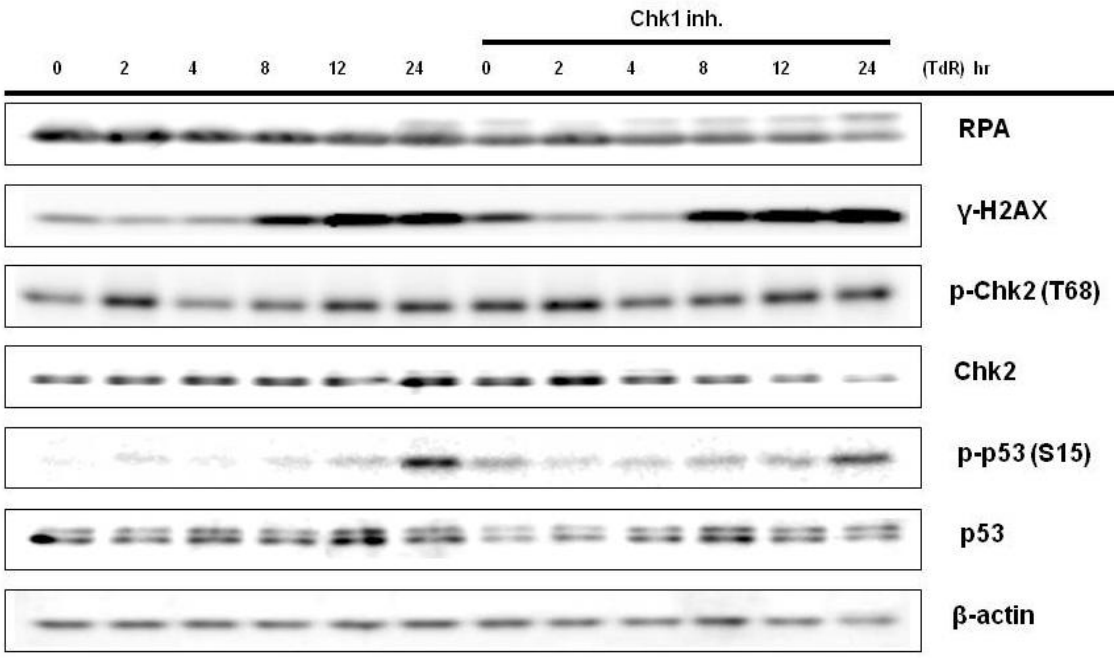



**FIGURE4.2B. Western blot analysis of DNA damage response proteins in EJ cells treated with thymidine in the presence or absence of CHK1 inhibitor.** EJ cells were exposed to 1mM thymidine for 0-24h in the presence or absence of 1μM Gö6976 before harvest and preparation of cell free extracts. Cells treated with Gö6976 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the CHK1 inhibitor alone (0h thymidine) were exposed for 24h. Western blots prepared from these extracts were analysed for the indicated damage response proteins. β-actin levels are presented as loading controls.

### 4.3.3 Apparent cell death is triggered by gemcitabine in CHK1 inhibited EJ cells upon DNA replication stress.

In addition to thymidine, the effect of gemcitabine was also examined in Gö6976 treated EJ cells. Here the EJ cells were treated with 12.5 and 25nM gemcitabine for 24hr in the presence or absence of a 2hr pre-incubation with Gö6976. Consistent with the response triggered by thymidine, the 24h co-treatment with gemcitabine and Gö6976 induced significant sub-$G_1$ DNA content in TCC-sup and EJ bladder cancer cells (Fig. 4.3). Notably, only gemcitabine or Gö6976 alone is enough to trigger death response in these two types of bladder tumour cells.

Taken together, in contrast to hTERT-NHU cells, these data clearly show that the combination of DNA replication stress agent and CHK1 inhibition are able to produce a strong commitment to apoptosis in bladder cancer cell lines.

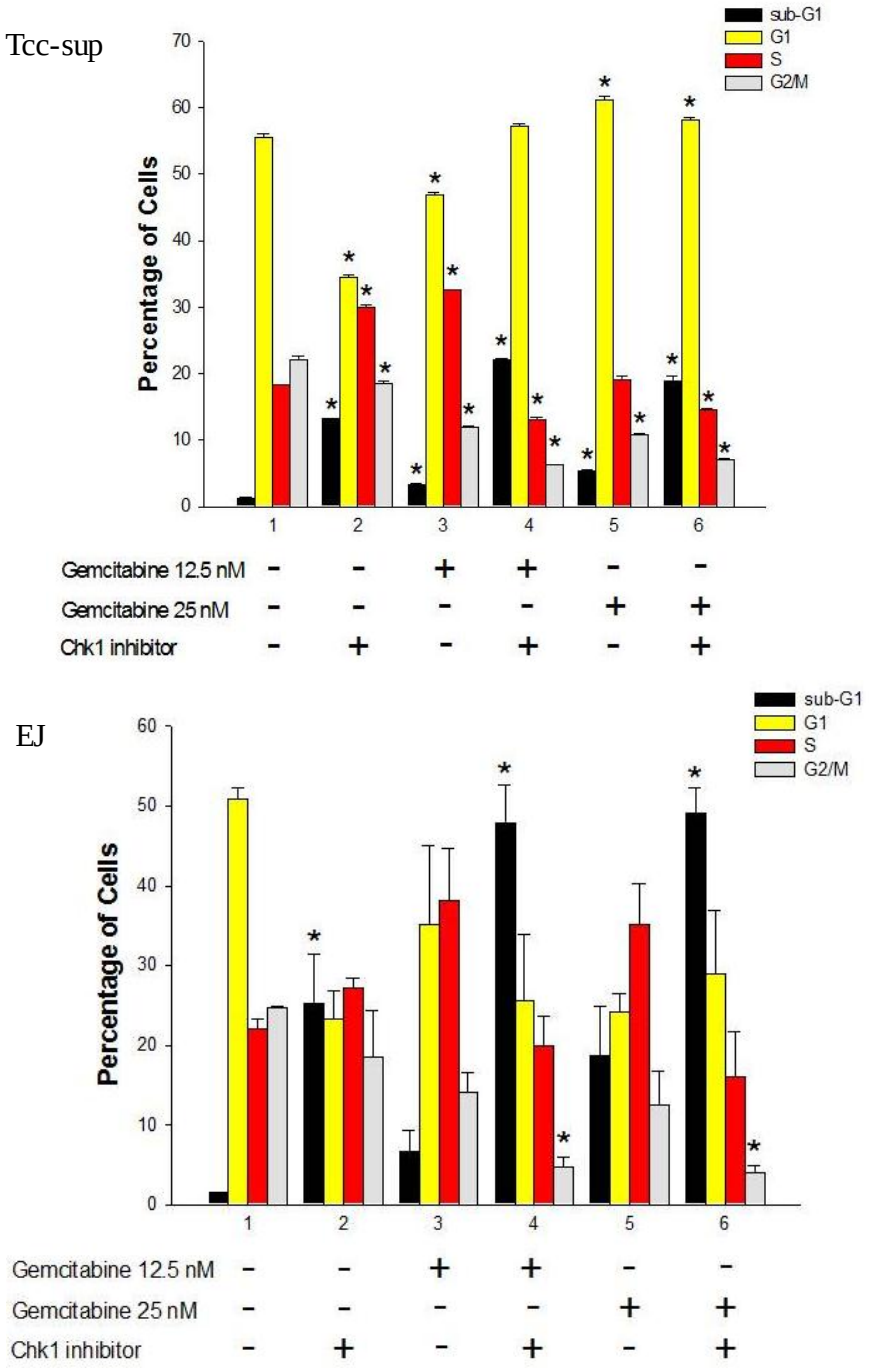



**FIGURE 4.3. Gemcitabine induces apoptosis in bladder cancer cell lines when CHK1 is inhibited.** TCC-sup and EJ bladder cancer cells were pre-treated with 1μM Gö6976 for 2 h and then incubated with or without 12.5nM Gemcitabine for 24h before harvest and analysis for cell cycle distribution by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

### 4.3.4 Neither thymidine nor gemcitabine induced significant death response in lower stage tumour cells combined with CHK1 inhibition.

In order to further examine the death response resulted from the combination of CHK1 deficiency and replication stress, more tumour cell lines including RT112, RT4, S637 and SW780 cell lines were screened as well. Compared with grade IV TCC-sup and invasive EJ cancer cells, these cell lines were much lower grade and not as malignant (RT4: papilloma, RT112: Grade I, SW780: Grade I, S637: Grade II) as TCC-sup and EJ according to neoplastic grading and TNM staging system. Similar to TCC-sup and EJ described previously, these bladder tumour cell lines were treated with thymidine in a dose-dependent manner for 24hr in the presence or absence of a 2hr pre-incubation with Gö6976. In contrast to Tcc-sup or EJ, only RT112 showed a small fraction of sub-$G_1$ DNA content resulted from the treatments compared with untreated control, no significant difference of the pattern of sub-$G_1$ population was observed in other cell lines (Fig. 4.4A).

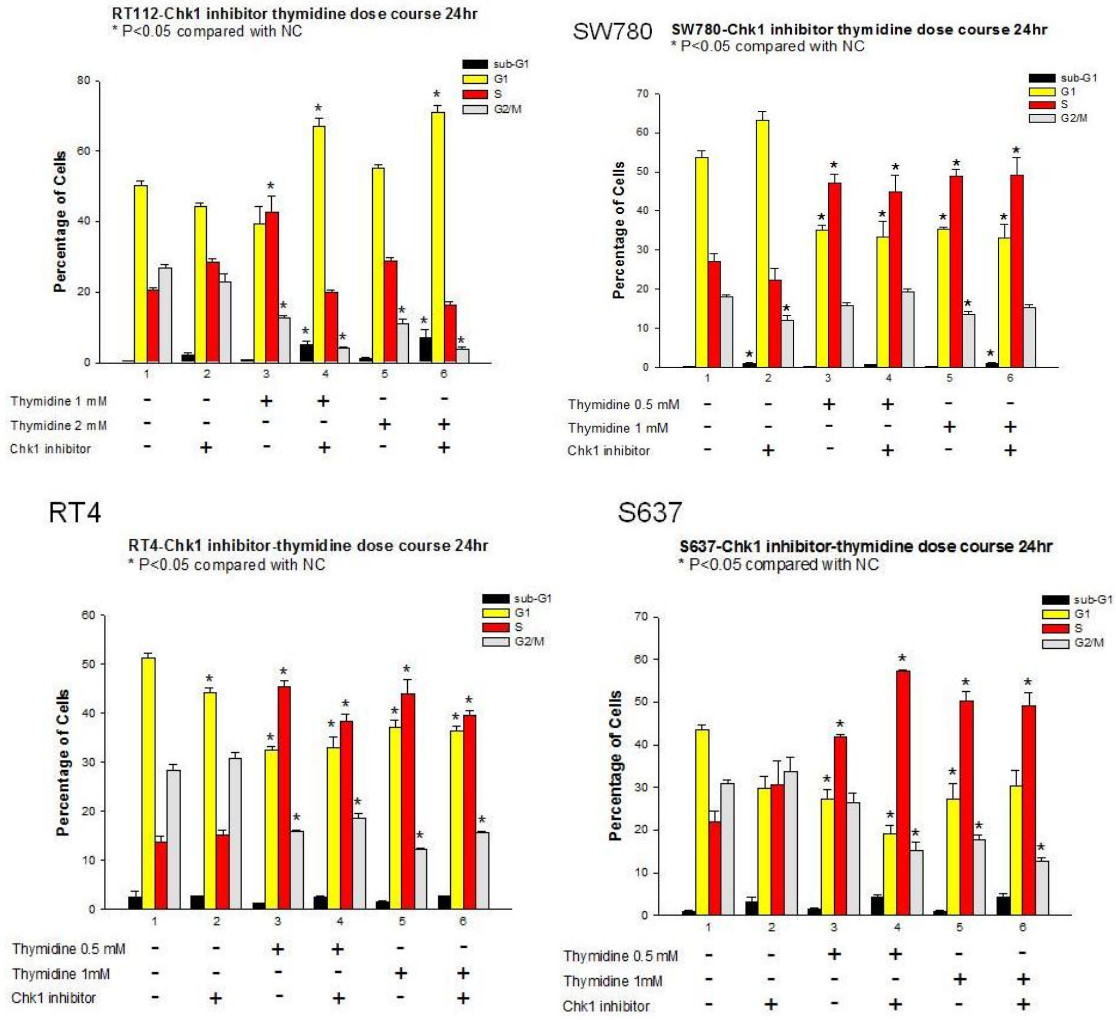



**FIGURE4.4A. Thymidine was unable to induce significant apoptosis responses in lower grade bladder cancer cell lines (except RT112) when CHK1 is inhibited.** Five different types of lower stage bladder cancer cell lines were pre-treated with 1μM Gö6976 for 2h and then incubated with or without thymidine as a dose-dependent manner for 24h before harvest and analysis for cell cycle distribution by flow cytometry. Bar graphs present the distributions of the cells in the different phases of the cell cycle. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells. Note: The induction of sub-$G_1$ populations in EJ cells serves as a positive control (presented as Fig. 4.1A)

Even when thymidine was replaced by the anti-cancer drug gemcitabine, only weak increases in apoptosis population was obtained in RT112 and RT4 cells when CHK1 was inhibited (Fig. 4.4B).

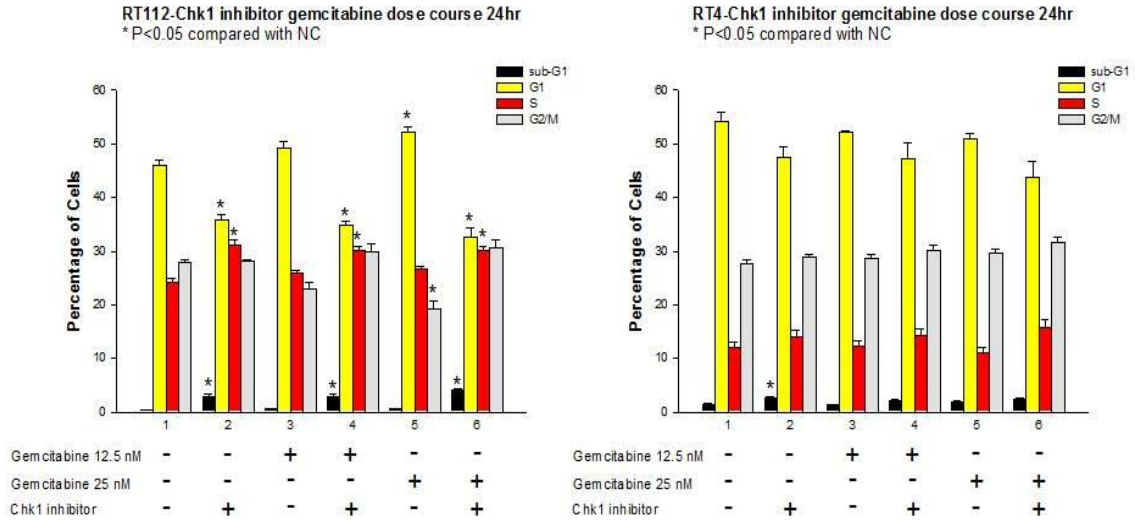



**FIGURE4.4B. Gemcitabine was unable to enhance apoptosis in RT112 and RT4 bladder cancer cell lines when CHK1 is inhibited.** RT112 (left) and RT4 (right) bladder cancer cells were pre-treated with 1μM Gö6976 for 2 h and then incubated with or without 12.5 and 25nM Gemcitabine for 24h before harvest and analysis for cell cycle distribution by flow cytometry. Bar graphs present the distributions of the cells in the different phases of the cell cycle. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells. Note: The induction of sub-$G_1$ populations in EJ cells serves as a positive control (presented as Fig. 4.3)

## 4.4 Discussion

It is widely agreed that cell cycle checkpoint systems regulate cell cycle progression in response to DNA damage or replication defects to ensure genome stability. Many laboratories have reported that CHK1 not only regulates S-phase progression in tumour cells but also protects them from apoptosis following DNA replication stress (Meuth, 2010; Petermann et al., 2006; Rodriguez and Meuth, 2006; Sidi et al., 2008). Consistently, this present study shows that the ATR/CHK1 pathway is activated in EJ and TCC-sup bladder cancer cells after treatment with replication inhibitor. Under replication stress CHK1seems dispensable for protection of these cells from apoptosis. However this survival dependence of CHK1 is not revealed in other bladder tumour cells. Why only EJ and TCC-sup show strong dependence on CHK1 for cell survival under replication stress is still not clearly understood. It is suggested that different degrees of malignancy might be the reason. While TCC-sup is ranked as a grade IV transitional cell carcinoma of urinary bladder, it is also reported that EJ or previously called MGH-U1 cells is a subline and have the same origin as T-24 (Lin et al., 1985; O'Toole et al., 1983) which is a cell line established from a high grade transitional cell bladder carcinoma in 1973 (Bubenik et al., 1973). In contrast, both RT4 and RT112 cells are only

well-differentiated, lowly proliferative and superficial grade I bladder cancer cell lines (Stravopodis et al., 2011). Similarly, the positive correlation between proliferation rate and drug sensitivity was also found in breast cancer that the clinical response of mostly DNA-targeted chemotherapy was significantly higher for rapidly than for slowly proliferating tomours (Amadori et al., 1997). Whether urothelial cells respond to chemotherapy via the similar mechanism needs more studies.

Furthermore, p53 status in tumour cells may also play a role in survival dependence of ATR-CHK1 in response to DNA replication stress. In mammalian cells, ATR-CHK1-Cdc25 signalling cascades cooperate with ATM-CHK2-p53 pathway to ensure genome integrity in response to DNA damage or replication stress (Goto et al., 2012). The p53 tumour suppressor gene has already been reported to correlate with cell cycle checkpoint and determine cell fate. Activated and stabilized p53 triggers cell cycle arrest, cell senescence or apoptosis by regulating its downstream proteins including the Cdk inhibitor p21 or the pro-apoptotic BAX and PUMA proteins (McGowan, 2002; Riley et al., 2008). In HCT116 colon cancer cells, cell survival was selectively reduced in $p53^{-/-}$ compared to $p53^{+/+}$ cells in response to the

combination of UCN-01-induced CHK1 inhibition and IR (Petersen et al., 2010). The similar effect of cell survival was also obtained in osteosarcoma U2OS-VP16 cells with and without expression of p53 dominant-negative mutation (Petersen et al., 2010). Dicoumarol, a naturally occurring anticoagulant was also reported to suppress p53/p21 and enhance cytotoxicity of doxorubicin via p38 MAPK pathway in RT112 urothelial cancer cells (Matsui et al., 2010). Thus, loss of functional p53 may lead cells to rely more heavily on the other signalling pathway, ATR-CHK1, for cell survival in response to DNA damages or replication defects.

It has been reported that most superficial urothelial cancers retain wild-type p53 and the mutation status were both associated with tumour grade and stage (Watanabe et al., 2004). Once p53 has mutated and lost its function, the ATR-CHK1 pathway then becomes the sole route of damage checkpoint in these cancer cells (Ma et al., 2011). This is consistent with the result described previously that only p53 mutated (EJ and TCC-sup) but not p53 wild-type bladder cancer cell lines (RT4, RT112, etc.) show prominent death response following DNA replication stress when CHK1 is inhibited. Taken together, it strongly suggests that p53-dependent cell cycle checkpoint may protect

human bladder cancer cells from cell death in response to the treatment of DNA replication and Chk1 inhibitors. However, since p53 is proven to be a tumour suppressor gene and highly correlated to cell apoptosis, why p53 deficient bladder tumour cell lines instead showed the prominent death response needs more studies.

# Chapter 5 ATM regulates $G_1$/S transition in normal urothelial cells and protects cell death in response to replication stress in tumour cells.

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# Chapter 5 ATM regulates $G_1$/S transition in normal urothelial cells and protects cell death in response to replication stress in tumour cells.

## 5.1 Introduction

ATM is a serine-threonine kinase (Abraham, 2001) with a large size of 370 KDa and a member of the phosphatidylinositiol 3-kinase family. The function of ATM is well characterised due to the research of the disease Ataxia Telangiectasia (AT) which is a rare autosomal recessive disease resulted from mutation of ATM gene. Patients with homozygous ATM mutations show an early onset of progressive ataxia alongside other neurodegenerative conditions causing difficulty with movement and coordination (Lavin and Shiloh, 1997). Mutated ATM genes in yeast, Drosophila and mammals also result in a variety of phenotypes with features similar to human AT cells (Savitsky et al., 1995). ATM deficiency leads to growth retardation, infertility and cancer-predisposition in mice (Barlow et al., 1996). ATM mutated cells are highly sensitive to ionising radiation (IR) due to the defect of $G_1$, S and $G_2$/M arrest (Painter and Young, 1980). Since ATM is immediately activated in response to DNA damage and involved in the regulation of broad downstream substrates, defects of ATM affect a wide range of cell fate such as apoptosis or

tumourigenesis due to its function in DNA repair, cell cycle checkpoint, gene regulation, translation initiation, and telomere maintenance (Kurz and Lees-Miller, 2004). It is generally accepted that ATR is activated due to the presence of single strand DNA (ssDNA) whereas ATM is triggered in response to DNA double strand breaks (DSBs). In response to DNA double strand breaks, ATM is recruited to the double strand ends by the three proteins Mre11, RAD50 and NBS1-formed complex (MRN complex) (Lee and Paull, 2007) and phosphorylates the histone H2AX on Ser139 (Huang et al., 2004). ATM-CHK2 also phosphorylates phosphatase CDC25, which inactivates CDK-Cyclin complex resulting in cell-cycle arrest (Matsuoka et al., 1998). If DNA DSBs are not repaired, ATM additionally phosphorylates MDM2 and p53 at Ser15 (Canman et al., 1998) leading to stabilization and activation of p53, which regulates numerous p53 target genes including Cdk inhibitor p21, proapoptotic proteins BAX, PUMA, and NOXA, and results in cell cycle arrest or apoptosis (Mirzayans et al., 2012). However, my lab has also taken the lead in demonstrating a novel role of ATM in response to DNA replication stress in the absence of DSBs. ATM deficient cells exposed to thymidine revealed a decrease of cell viability and failed to trigger homologous recombination repair (HRR) (Bolderson et al., 2004).

## 5.2 Aims and Hypothesis

The generally accepted model is that ATR and ATM activated as an early regulator of either DNA replication arrest or DNA double strand breaks. In the previous chapters, the apparently distinct roles of ATR-CHK1 pathway was examined in normal human urothelial (NHU) cells versus bladder cancer cells. The ATR-CHK1 pathway is not fully activated in hTERT-NHU cells and only modestly affected after treatment with replication stressors. Since ATM and its downstream protein Chk2 are considered as regulators which participate in a pathway in parallel to ATR (Cliby et al., 1998) and shares some overlapping function such as Cdc25A phosphorylation (Falck et al., 2001; Zhao et al., 2002), it is worthwhile to examine the relationship between ATM and ATR pathways particularly in DNA replication stress-induced checkpoint system. A hypothesis was proposed that ATM replaces ATR-CHK1 and acts as the major regulator in cell cycle progression in normal urothelial cells following replication defects. Treatments-induced protein phophorylation and total level changes were examined by Western blot. The role of ATM in cell cycle progression in response to replication stress was studied by FACS flow cytometry. DNA damage response proteins were tested by not only immunoblots but also immunofluorescence.

## 5.3 Results

### 5.3.1 ATM is activated early by DNA replication stress in hTERT-NHU cells.

To determine whether ATM is also activated in hTERT-NHU cells in response to replication inhibitors, cultures were treated with thymidine in the presence or absence of Gö6976 for different time points and harvested for Western blot analysis. Interestingly, in contrast to CHK1 described previously, ATM was autophosphorylated (at Ser1981) very early by 2hr after exposed to thymidine (Fig. 5.1).

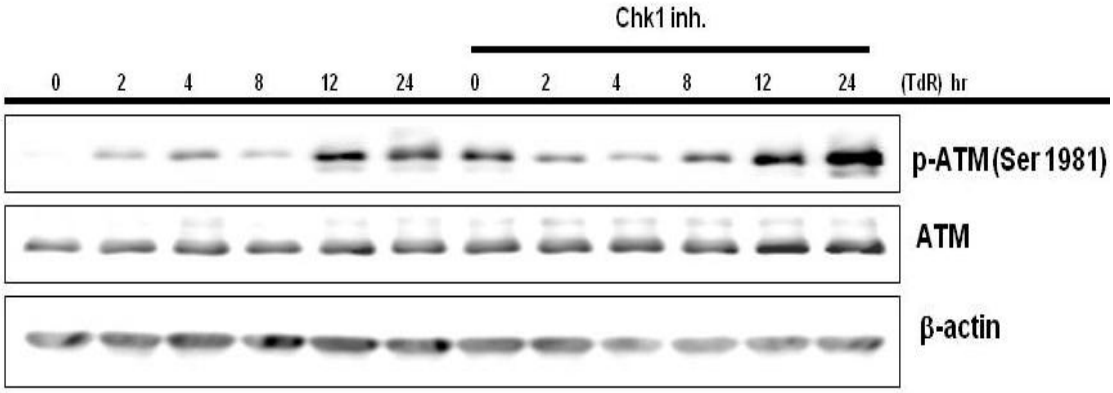



**FIGURE5.1. Western blot analysis of ATM activation in hTERT-NHU cells treated with thymidine in the presence or absence of CHK1 inhibitor.** hTERT-NHU cells were exposed to 1mM thymidine for 0-24h in the presence or absence of 1μM Gö6976 before harvest and preparation of cell free extracts. Cells treated with Gö6976 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the CHK1 inhibitor alone (0h thymidine) were exposed for 24h. Western blots prepared from these extracts were analysed for ATM activation. β-actin levels are presented as loading controls.

Downstream phosphorylation targets of activated ATM (pThr68 CHK2 and pSer15 p53) were also examined in hTERT-NHU cells after exposed to thymidine treatment as described previously (Fig. 3.3B, 3.3C). These events look independent of thymidine-induced CHK1 activation as they were not affected by Gö6976 caused CHK1 inhibition.

### 5.3.2 ATM kinase activity is required for $G_1$-S phase transition in hTERT-NHU cells but not S-phase progression.

Since ATM showed the relatively early activation following thymidine treatment in the presence or absence of Chk1 inhibitor, the effect of ATM inhibition on immortalized normal urothelial cells during DNA replication stress was then examined. hTERT-NHU cells were firstly treated with 10 μM of the highly specific ATM inhibitor KU55933 (Hickson et al., 2004) for 2 hr and then incubated in the presence or absence of thymidine or gemcitabine for 24hr. Cells were harvested for cell cycle analysis by FACS. As expected, there was almost no cell death detected due to the treatment. Interestingly, the fraction of cells in S phase was significantly reduced following KU55933 treatment no matter in the presence or absence of thymidine or gemcitabine relative to untreated control (Fig. 5.2A, B). This response was further confirmed in primary NHU cells. Primary NHU cells showed a similar reduction of S-phase population following treated with KU55933 in both presence or absence of thymidine treatment (Fig. 5.2C). Additionally, a second ATM specific kinase inhibitor resulted in similar results with reduced S-phase fraction (Fig. 5.2D).

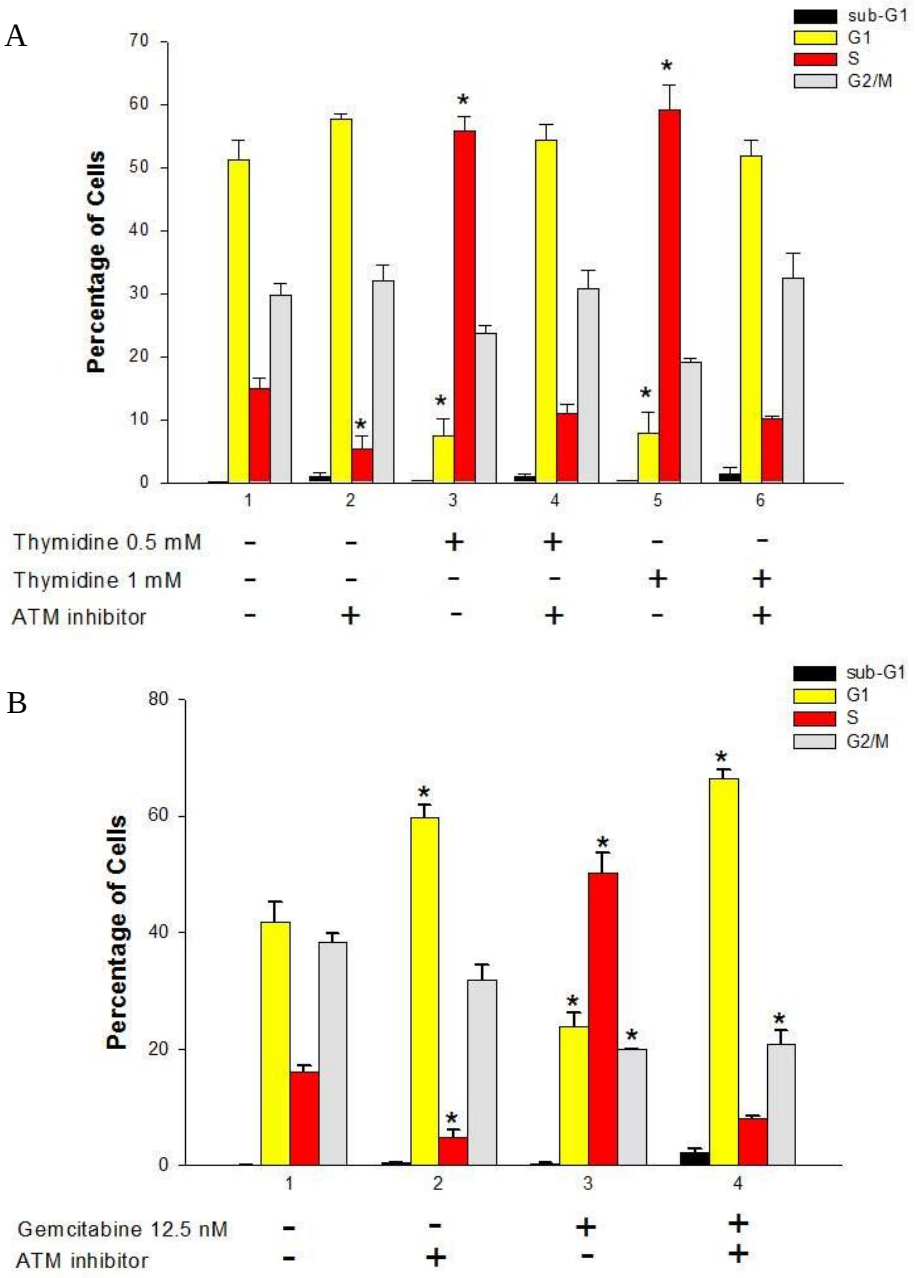



**FIGURE5.2. Active ATM is essential for $G_1$ to S phase progression in hTERT-NHU cells.** hTERT-NHU cells were pre-incubated with 10μM KU55933 for two hours before addition of 1mM thymidine (A) or 12.5nM gemcitabine (B). After 24h cells were harvested for analysis by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

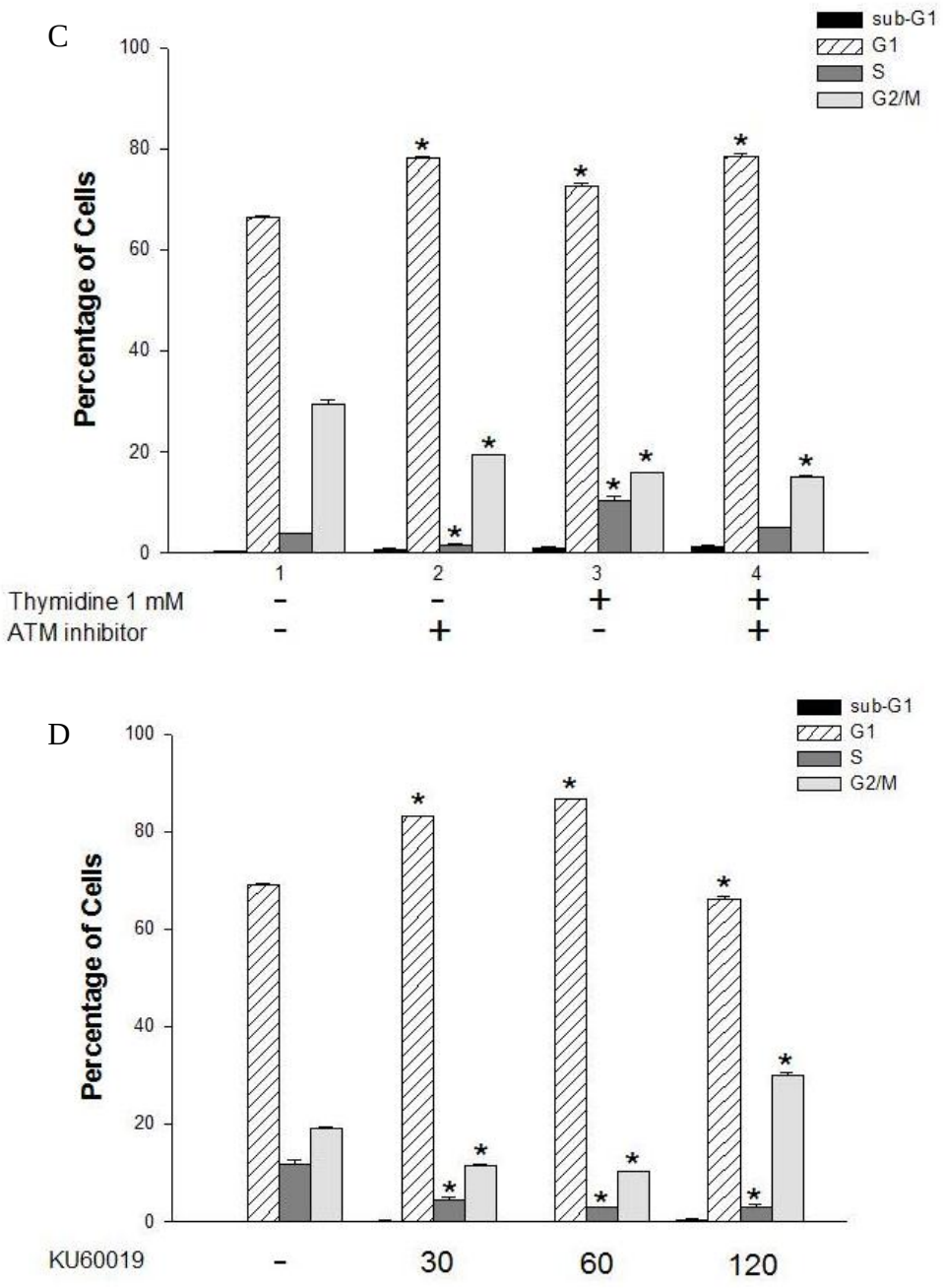



**FIGURE5.3. Active ATM is essential for $G_1$ to S phase progression in hTERT-NHU cells.** (C) Primary NHU cells were pre-incubated with 10μM KU55933 for two hours before addition of 1mM thymidine. After 24h cells were harvested for analysis by flow cytometry. (D) hTERT-NHU cells were treated with KU60019 as dose dependent manner. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

To further determine whether the effect of the ATM inhibitor was restricted to cells entering S-phase, hTERT-NHU cells were first treated with KU55933 and then incubated with the mitotic inhibitor nocodazole to trap cycling cells entering $G_2$/M phase. As shown in the results, the combination of ATM inhibitor and nocodazole not only reduced the level of S-phase cells but also increased the number in $G_2$/M relative to untreated control (Fig. 5.3). This may due to the fact that the cells already in S-phase successively progressed into $G_2$/M where they were arrested by nocodazole treatment and no new cells were able to enter S phase.

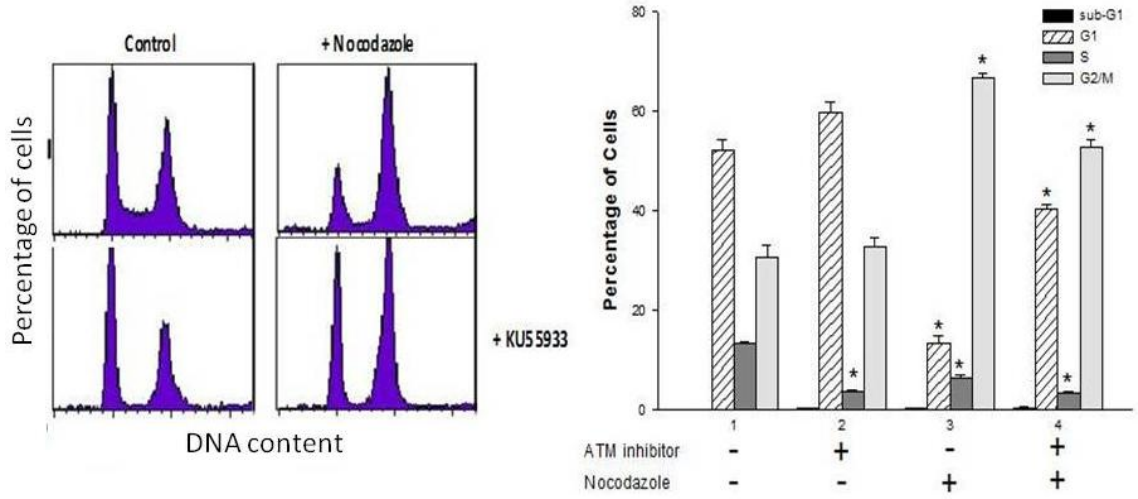



**FIGURE5.4. Effect of Nocodazole on hTERT-NHU cells in the presence or absence of ATM inhibitor.** hTERT-NHU cells were treated with nocodazole (right panels) in the presence or absence of 10 μM KU55933 (bottom panels) for 24h before harvest and analysis for DNA content by flow cytometry. Bar graph shows the mean values of the percentages of cells in the different phases of the cell cycle. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

Consistently, the fraction of cells incorporating BrdU indicated that they had difficulty entering S-phase with very low levels of $BrdU^+$ cells in response to the treatment of KU55933 particularly by 24hr (Fig. 5.4).

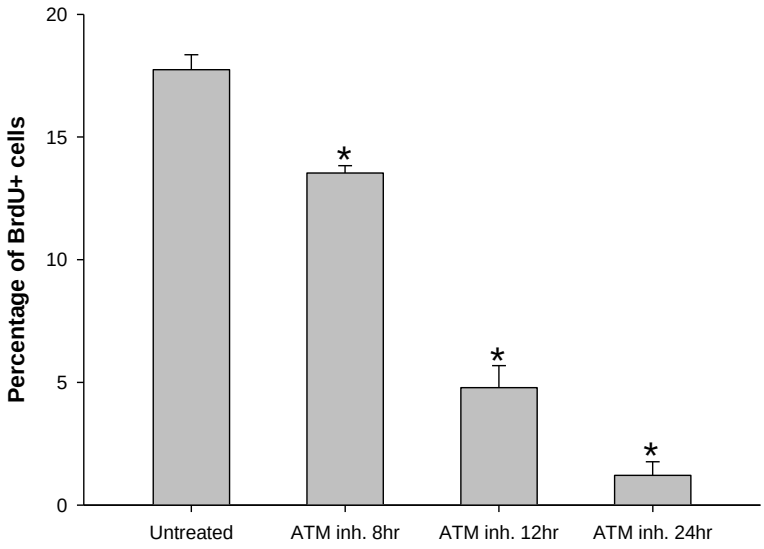



**FIGURE5.5. Analysis of BrdU incorporation on ATM inhibitor pre-treated hTERT-NHU cells.** hTERT-NHU clls treated with KU55933 for the indicated times were labelled with BrdU for 2h before harvest and analysis of BrdU incorporation by flow cytometry. Values presented represent the mean of at least three independent experiments ±standard deviation. * $P<0.05$ compared with untreated negative control (NC) cells.

hTERT-NHU cells were also pre-labelled with BrdU for 2h followed by addition of ATM inhibitor to examine the fate of cells already in S-phase. Cells were then harvested 24h after addition of KU55933 and analysed for both DNA content and BrdU incorporation. In both groups with presence or absence of the ATM inhibitor, labelled cells successively progressed through S-phase and

they were present in both $G_2$/M and $G_1$ after 24hr suggesting that the ATM inhibitor had little effect on S or $G_2$/M phase. However, almost no BrdU incorporation could be found in S-phase cells once ATM was inhibited for 24hr in hTERT-NHU cells indicating that ATM inhibition triggered $G_1$ phase checkpoint and blocked S-phase entry (Fig. 5.5).

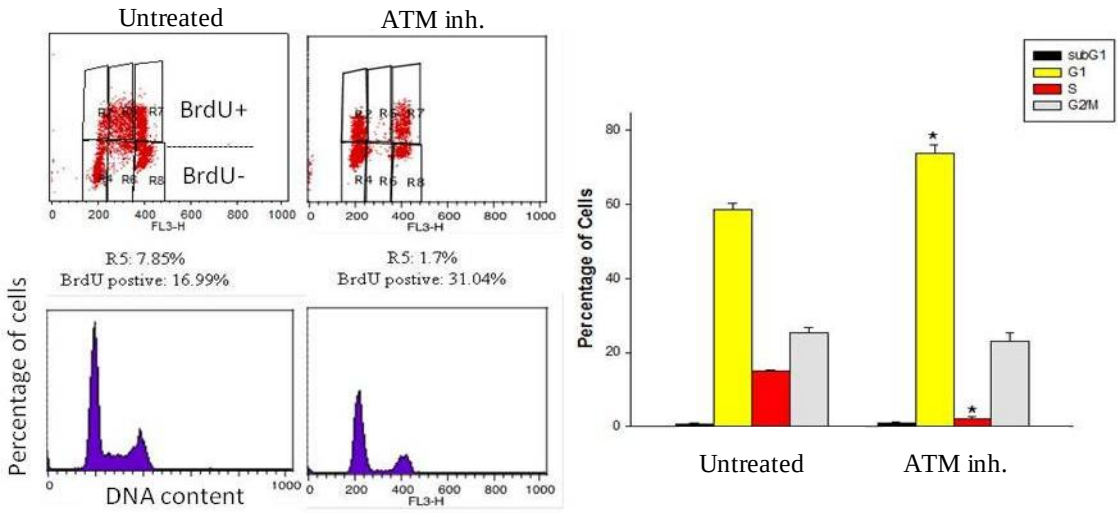



**FIGURE5.6. The ATM inhibitor does not stop progression of hTERT-NHU cells through S- or $G_2$/M phases.** hTERT-NHU cells pre-labelled with BrdU (10mM) were incubated in the presence or absence of KU55933 (10μM) for 24h. Cells were then harvested and analysed for BrdU incorporation and DNA content. Cells in S-phase at the time of BrdU labelling progressed through S-phase and into $G_2$/M and $G_1$ in the presence or absence of the ATM inhibitor. Values presented represent the mean of at least three independent experiments ±standard deviation. * $P<0.05$ compared with untreated negative control (NC) cells.

Taken together, these data indicate that ATM kinase activity is required for S-phase entry in hTERT-NHU cells but not for progression through S- or $G_2$/M-phases.

### 5.3.3 ATM kinase plays a role upstream of CHK1 checkpoint pathway.

Alteration of protein activation and total protein levels in response to ATM inhibition were also examined. Western blot analysis of extracts prepared from hTERT-NHU cultures showed that the ATM inhibitor KU55933 suppressed the thymidine treatment –induced autophosphorylation of ATM at Ser1981 confirming the effectiveness of the inhibitor (Fig. 5.6A).

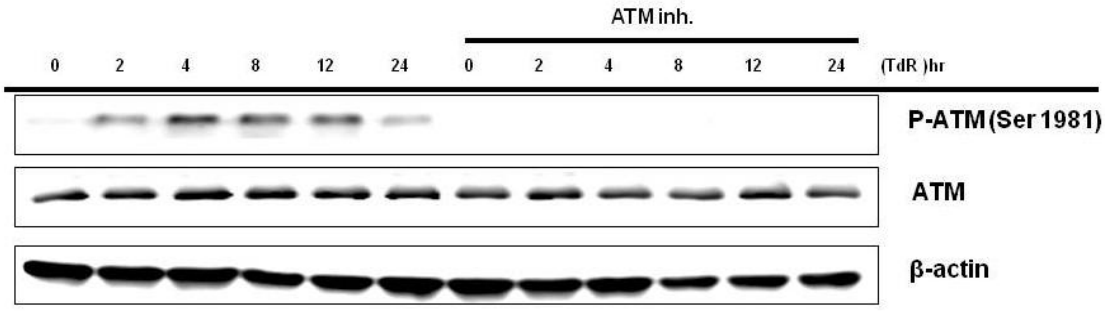



**FIGURE5.7A. Analysis of ATM activation in hTERT-NHU cells treated with thymidine in the presence or absence of the ATM inhibitor.** hTERT-NHU cells were incubated from 0-24h with 1mM thymidine in the presence or absence of 10μM KU55933 before harvest and preparation of cell free extracts. Cells treated with KU55933 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the ATM inhibitor alone (0h thymidine) were exposed for 24h. Western blots prepared from these extracts were analysed for the activated and total protein level of ATM. β-actin levels are presented as loading controls.

Notably, even though total Chk1 level did not seem to down regulate in response to ATM inhibition, thymidine-induced Ser317 and Ser296 Chk1 phosphorylation were suppressed after treatment with KU55933 suggesting

that ATM activation may play a role upstream of Chk1, which regulates Chk1 phosphorylation. Furthermore, Ser15 phosphorylated forms of p53 and protein level also decreased under these conditions relative to cells treated with thymidine alone. Unlike CHK1 inhibited hTERT-NHU cells, thymidine treated hTERT-NHU cells failed to phosphorylate RPA and γ-H2AX following addition of ATM inhibitor KU55933 by 24h (Fig. 5.6B).

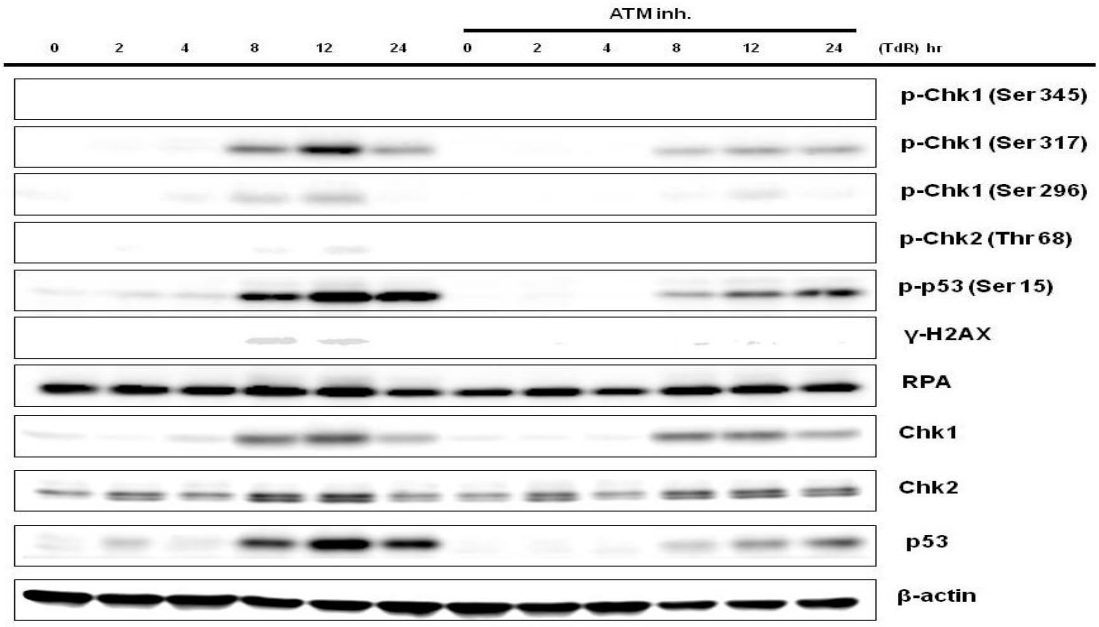



**FIGURE5.6B. Analysis of DNA damage response and cell cycle regulatory proteins in hTERT-NHU cells treated with thymidine in the presence or absence of the ATM inhibitor.** hTERT-NHU cells were incubated from 0-24h with 1mM thymidine in the presence or absence of 10μM KU55933 before harvest and preparation of cell free extracts. Cells treated with KU55933 were pre-incubated with the inhibitor for 2h before addition of thymidine, those treated with the ATM inhibitor alone (0h thymidine) were exposed for 24h. Western blots prepared from these extracts were analysed for the indicated damage response proteins. β-actin levels are presented as loading controls. Note: Expression of p-Chk1 Ser345 in EJ cells serves as a positive control (presented as Fig. 4.2A, Chapter 4).

### 5.3.4 RPA or γ-H2AX foci were not induced in hTERT-NHU cells treated with ATM inhibitor.

Additionally, in consistent with the Western blot analysis described previously (Fig. 5.6B, 5.7A), there was no further increase of cells which formed RPA or γ-H2AX foci in response to the combined treatment of the two agents relative to cells treated with thymidine or KU55933 alone (Fig. 5.7B). Compared with chemical inhibition of the kinase inhibitor, HCT116 was considered as a positive control with its siRNA-caused cellular protein depletion.

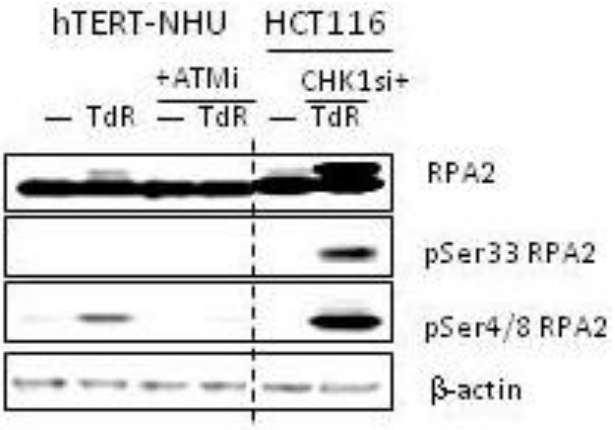



**FIGURE5.8A. Western blot analysis of RPA phosphorylation in hTERT-NHU cells treated with thymidine in the presence or absence of ATM inhibitor.** Western blot analysis of phosphorylated forms of RPA2 in extracts prepared from hTERT-NHU cells treated with 1mM thymidine for 24h with or without KU55933 (10μM). HCT116 cells treated with CHK1 siRNA and thymidine were generously gifted by Dr. Zuazua-Villar and presented as a positive control for full hyperphosphorylation of RPA2 (Gagou et al., 2010; Rodriguez et al., 2008).

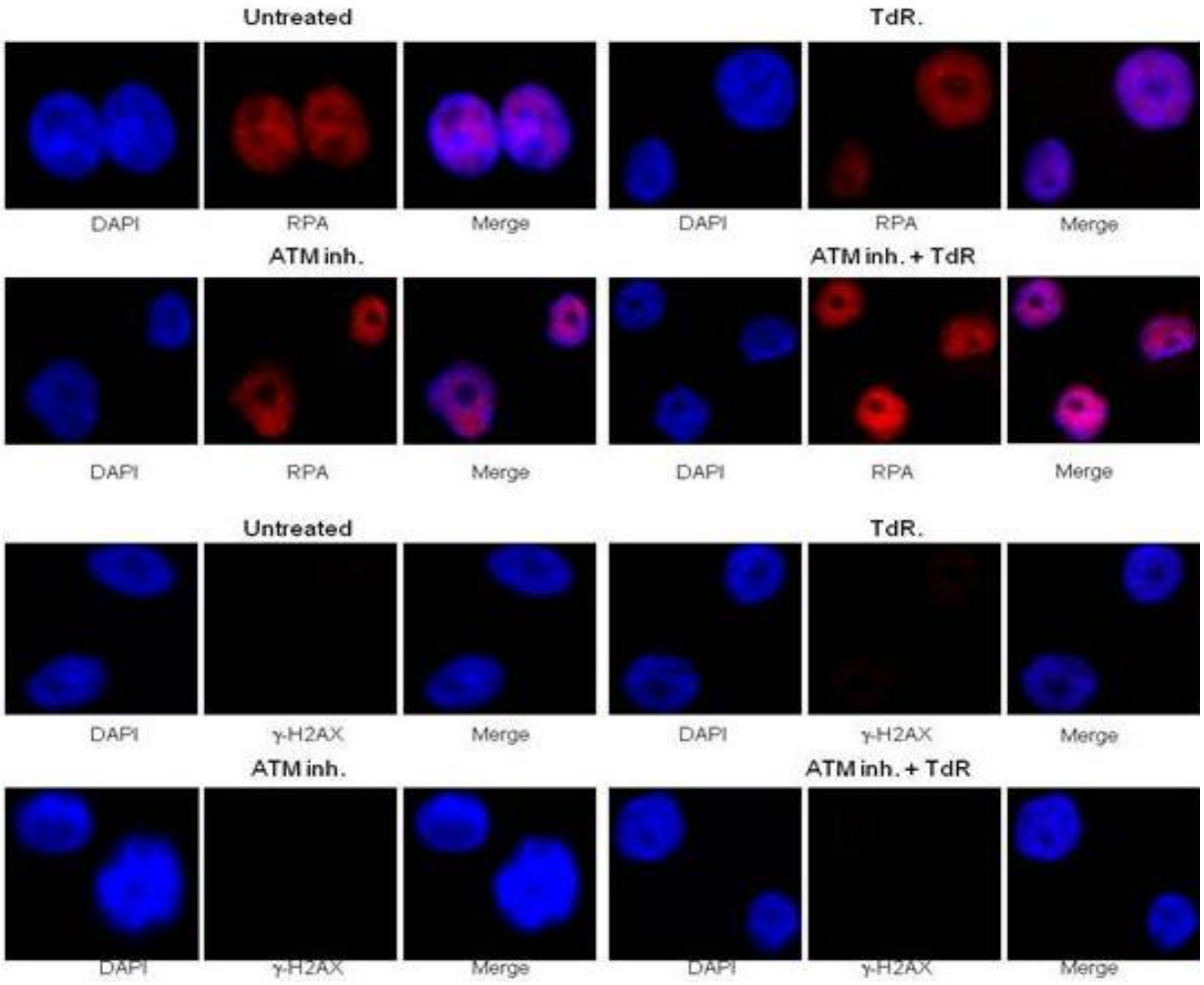
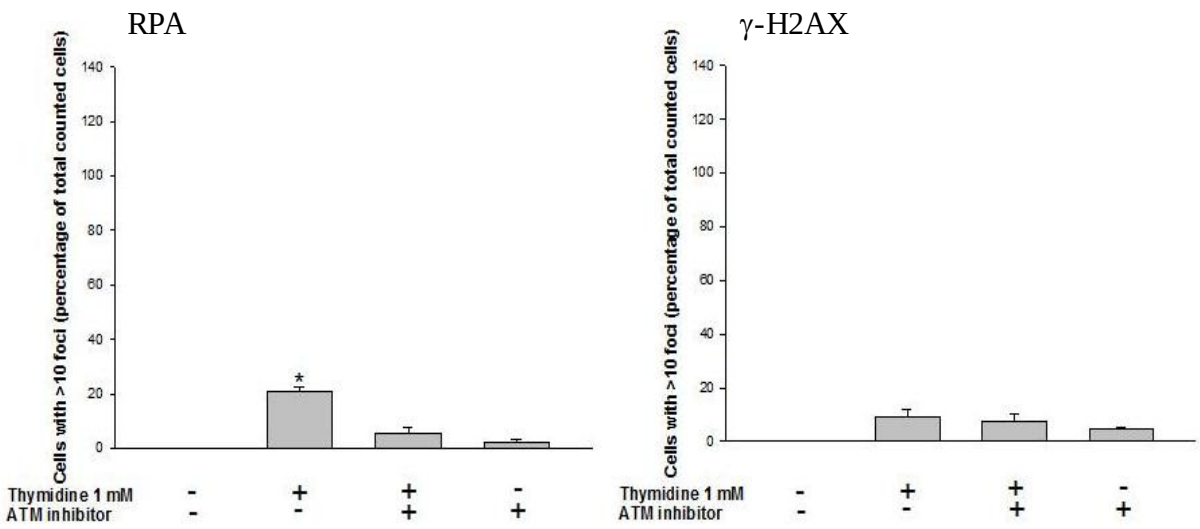



**FIGURE5.7B. Immunofluorescence analysis of RPA and γ-H2AX foci in hTERT-NHU cells treated with thymidine in the presence or absence of ATM inhibitor.** Representative images from immunofluorescence analysis of RPA and γ-H2AX foci in hTERT-NHU cells treated with 1mM thymidine and 1μM KU55933.




Bar graphs present the mean of at least three independent experiments ±standard deviation of hTERT-NHU cells treated with 1mM thymidine for 24h with or without KU59935 (10μM). Foci images of hTERT-NHU cells treated with Chk1 inhibitor and thymidine serve as positive controls (presented as Fig. 3.4, Chapter3).

Furthermore, the fraction of cells forming RAD51 foci following thymidine treatment was significantly reduced by treating with ATM inhibitor (Fig. 5.7C). These results strongly suggest that ATM is necessary for S-phase entry and consistent with the conclusion that hTERT-NHU cells fail to enter S-phase after treatment with the ATM inhibitor.

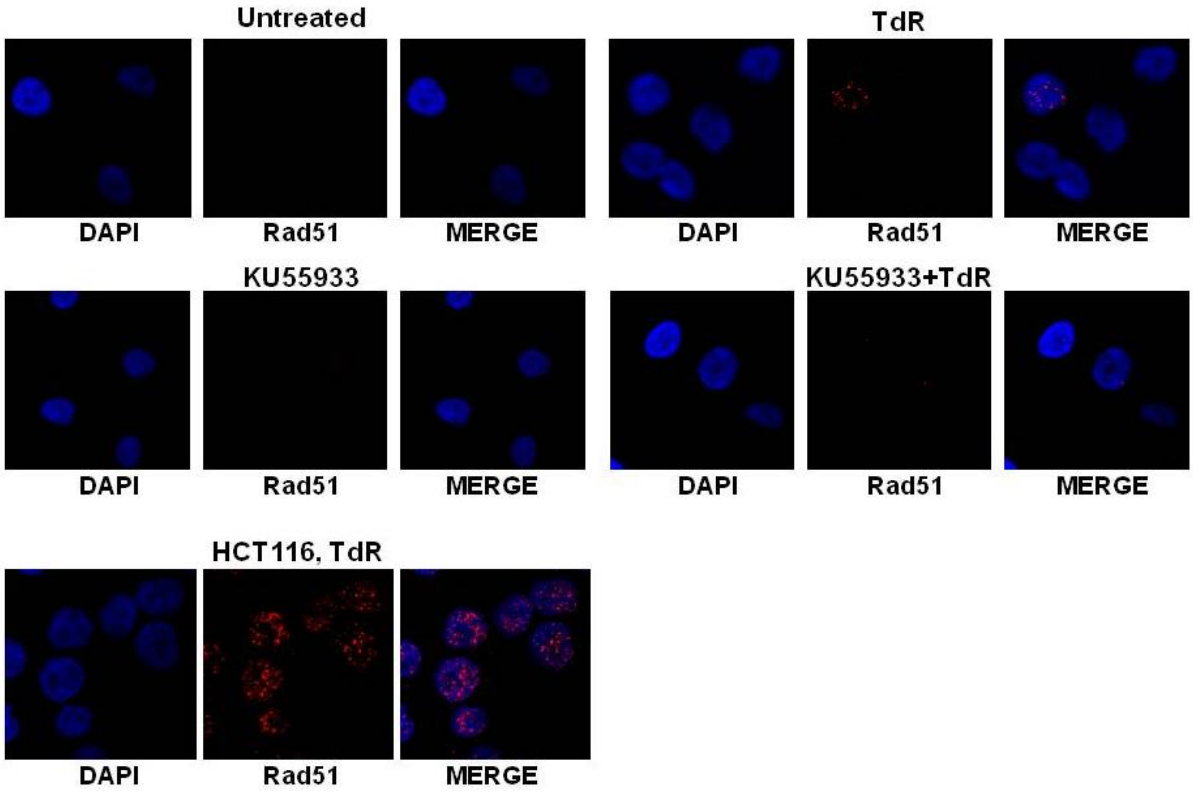
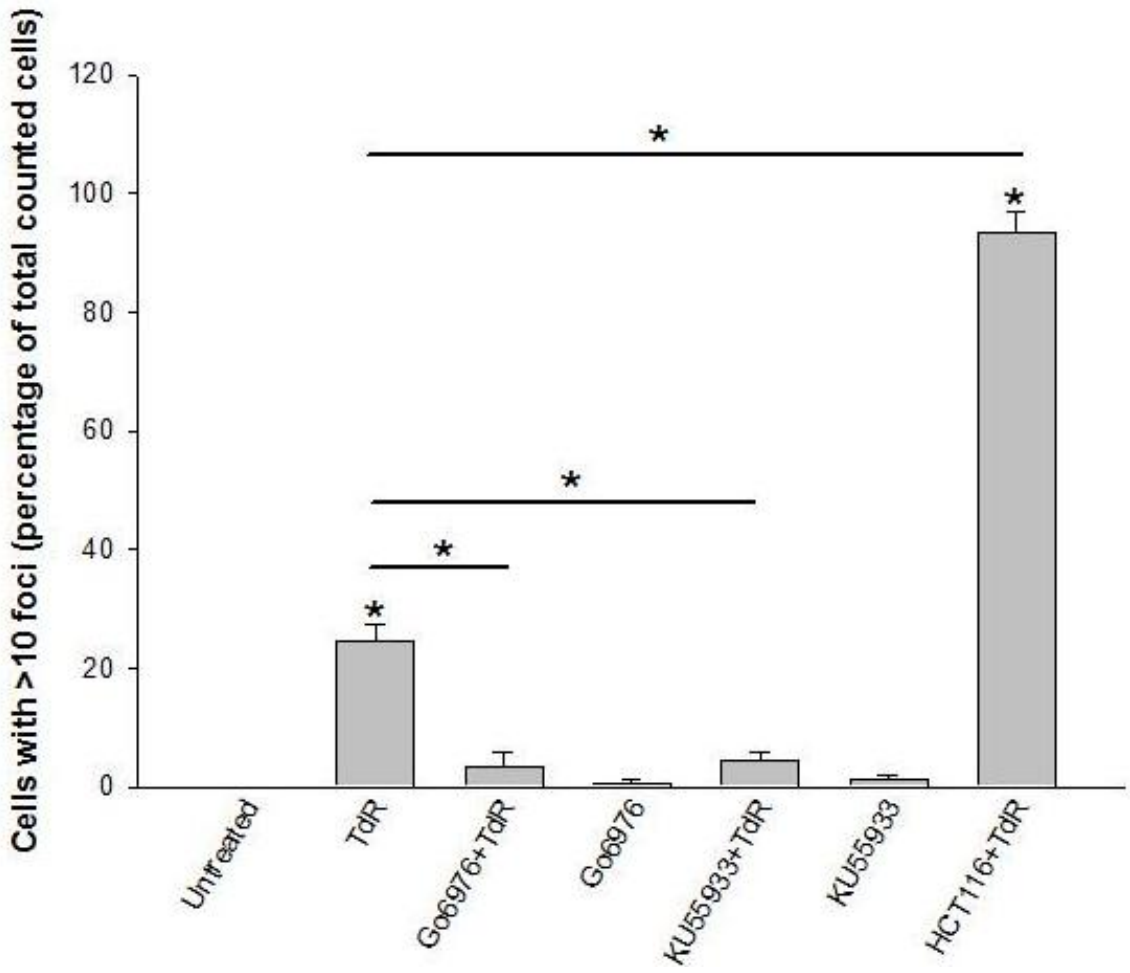



**FIGURE5.7C. Suppression of RAD51 foci formation by ATM inhibition in hTERT-NHU cells following treatment with thymidine.** hTERT-NHU or HCT116 cells treated with 1mM thymidine in the presence or absence of the ATM inhibitor KU55933 (10μM) for 24h were fixed and stained for DNA content and RAD51. Images from immunofluorescence analysis represent RAD51 foci in hTERT-NHU or HCT116 cells treated with thymidine.




The percentages of cells showing >10 RAD51 foci were scored. Bar graphs present the mean of at least three independent experiments ±standard deviation of hTERT-NHU cells or HCT116 cells.

### 5.3.5 ATM inhibition triggers a $G_1$ checkpoint hTERT-NHU cells.

How ATM inhibition regulates the S phase entry was next examined. In 2004 there is a paper reported that ATM normally controls the generation of reactive oxygen species (ROS). If ATM function is lost, p16*INK4a* is induced by ROS and this inhibits the CDK-mediated phosphorylation of retinoblastoma (RB) necessary for the S entry. Thus ATM inhibition triggers a $G_1$ checkpoint that can be relieved by treatment with ROS inhibitors (Ito et al., 2004). To determine whether a similar mechanism was responsible for $G_1$-S phase transition, the ROS inhibitor N-Acetylcysteine (NAC) was tested in hTERT-NHU cells. However ATM suppression-induced block of S-phase entry was not rescued by the treatment of NAC in hTERT-NHU cells (Fig. 5.8).

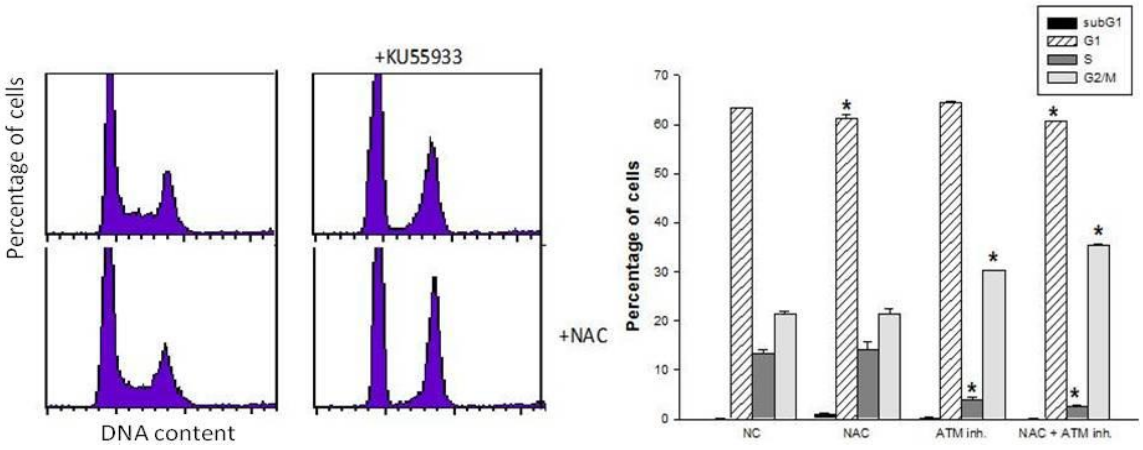



**FIGURE5.9. Induction of $G_1$ arrest by the ATM inhibitor in hTERT-NHU cells is not rescued by an ROS inhibitor.** hTERT-NHU cells exposed to the ATM inhibitor for 24h in the presence or absence of the ROS inhibitor N-acetylcysteine (100μM) were harvested for analysis of cell cycle distribution by flow cytometry. A representative profile of such an analysis is presented together with a bar graph presenting the distributions of the cells in the different phases of the cell cycle. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

To further examine whether the failure of hTERT-NHU cells to enter S-phase was due to an induction of a $G_1$ checkpoint, $G_1$ checkpoint associated proteins were analysed in cells treated with ATM inhibitor. As shown in the result, levels of $p16^{INK4a}$ did not show any apparent change following exposure to KU55933 in the presence or absence of thymidine (Fig. 5.9), however the level of another cyclin-dependent kinase inhibitor $p19^{INK4D}$ appeared an dramatic increase and the CDK4/6-mediated phosphorylation of RB that is normally triggered when cells entering S-phase was reduced in cells following treated with KU55933. Additionally Cyclin D1 and CDK4 levels were down regulated in

cells treated with KU55933, which is consistent with an arrest in $G_1$ (Baldin et al., 1993). In contrast, the level of another CDK-inhibitor (CDKN1A, p21) was not increased in cells treated with the ATM inhibitor (Fig. 5.9). Taken together these results suggest that hTERT-NHU cells fail to enter S-phase after treatment with the ATM inhibitor as a result of the activation of a $G_1$ checkpoint.

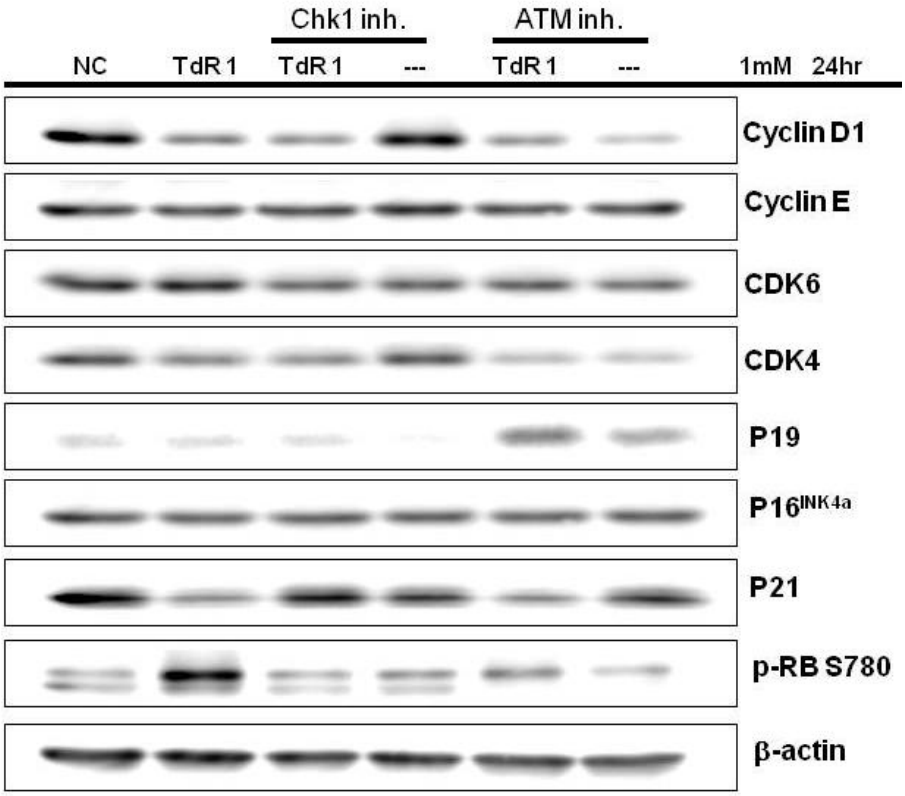



**FIGURE5.10. Analysis of cell cycle regulatory proteins in hTERT-NHU cells treated with thymidine in the presence or absence of the ATM inhibitor.** hTERT-NHU cells treated with 10μM KU55933 or 1μM Gö6976 inhibitors in the presence or absence of 1mM thymidine for 24h were harvested and analysed for the indicated cell cycle regulators by Western blotting. β-actin levels are presented as loading controls.

### 5.3.6 EJ bladder cancer cells show enhanced apoptosis after treatment with the ATM inhibitor and thymidine.

The effect of ATM inhibition on malignant bladder cancer cells was also examined. When EJ cancer cells were treated with KU55933 alone, the population of S-phase cells significantly increased compared to untreated cells however only few cells with sub-$G_1$ DNA content were detected (Fig. 5.10A). In response to combined treatment of KU55933 and thymidine, the fraction of cells with a sub-$G_1$ DNA content increased dramatically accompanied by a decreased level of S- and $G_2$/M-phase cells. This sign of cell death was further confirmed by activation of caspase3 (Fig. 5.10A, 5.10B) following the combination of KU55933 and thymidine treatment compared to cells treated with thymidine alone. Thus, in striking contrast to the hTERT-NHU cells described previously, ATM inhibition not only slows cell cycle progression through S-phase but also strongly commits EJ cells to apoptosis in response to DNA replication stress.

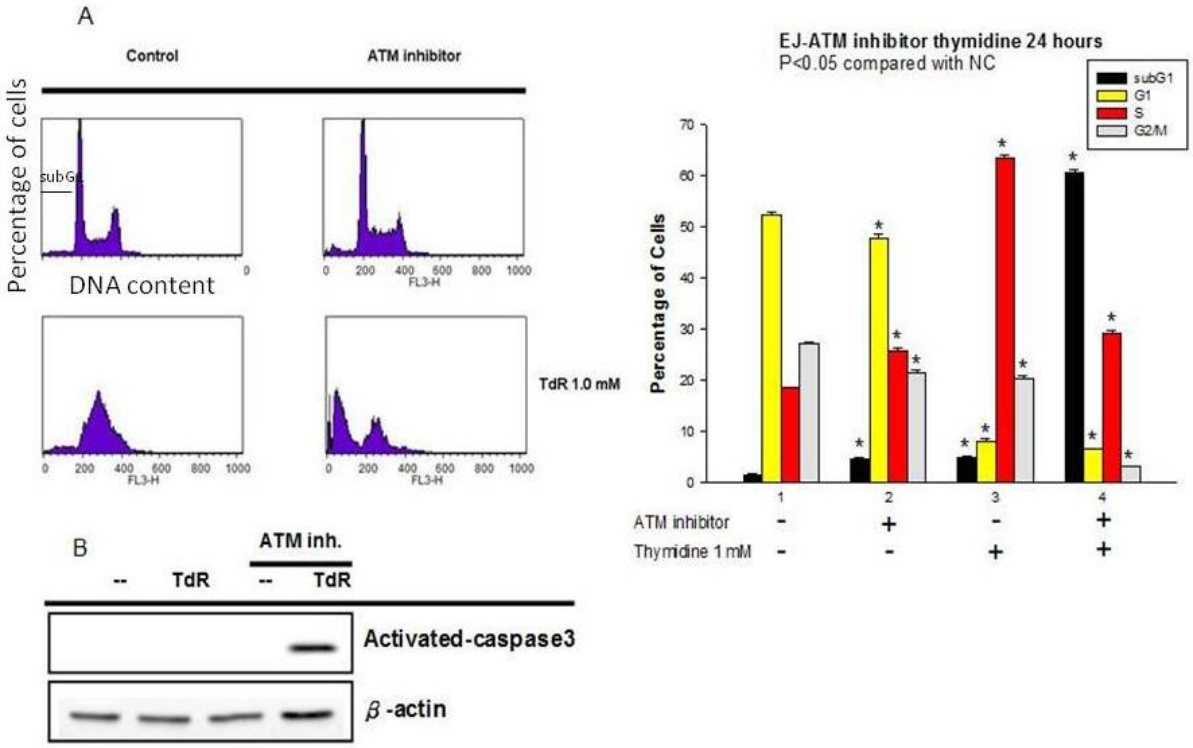



**FIGURE5.11. Active ATM protects EJ bladder cancer cells from death during replication stress.** (A) EJ bladder cancer cells were pre-incubated with KU55933 (10μM) for two hours before addition of 1mM thymidine. Cells were harvested at 24h for analysis by flow cytometry. (B) Western blot analysis of EJ cells treated as above for caspase3 activation.

### 5.3.7 DNA replication was able to restart in hTERT-NHU cells when they were released from CHK1 or ATM inhibition.

In this study, the combination of CHK1/ATM inhibition and DNA replication were demonstrated to be able to selectively kill tumour cells with only some slight alteration on normal urothelial cells. So far there are many reports claimed that their “targets” of cancer therapy are able to specifically kill tumour cells, however data were shown that most of those treatments exists some side effects such as irreversible cell senescence on non-tumour cells. Thus, it is important to see whether the effects on non-targeted cells were reversible or not. hTERT-NHU cells were pre-incubated with CHK1/ATM inhibitor for 2h followed by a 24h thymidine treatment. Cells were then released from treatments with fresh medium for another 24h and pulsed with BrdU for 2h before harvest and analysis of BrdU incorporation and DNA content by FACS. Here the treatment-induced cell cycle alteration seems able to be restored once they escaped from the inhibition. Although cells treated with thymidine in the presence of the CHK1 inhibitor show a little evidence of cell cycle re-entry, cells treated with ATM, CHK1 or replication inhibitors alone were demonstrated largely able to resume DNA replication particularly those released from ATM inhibition showed very high S phase DNA content (Fig. 5.11).

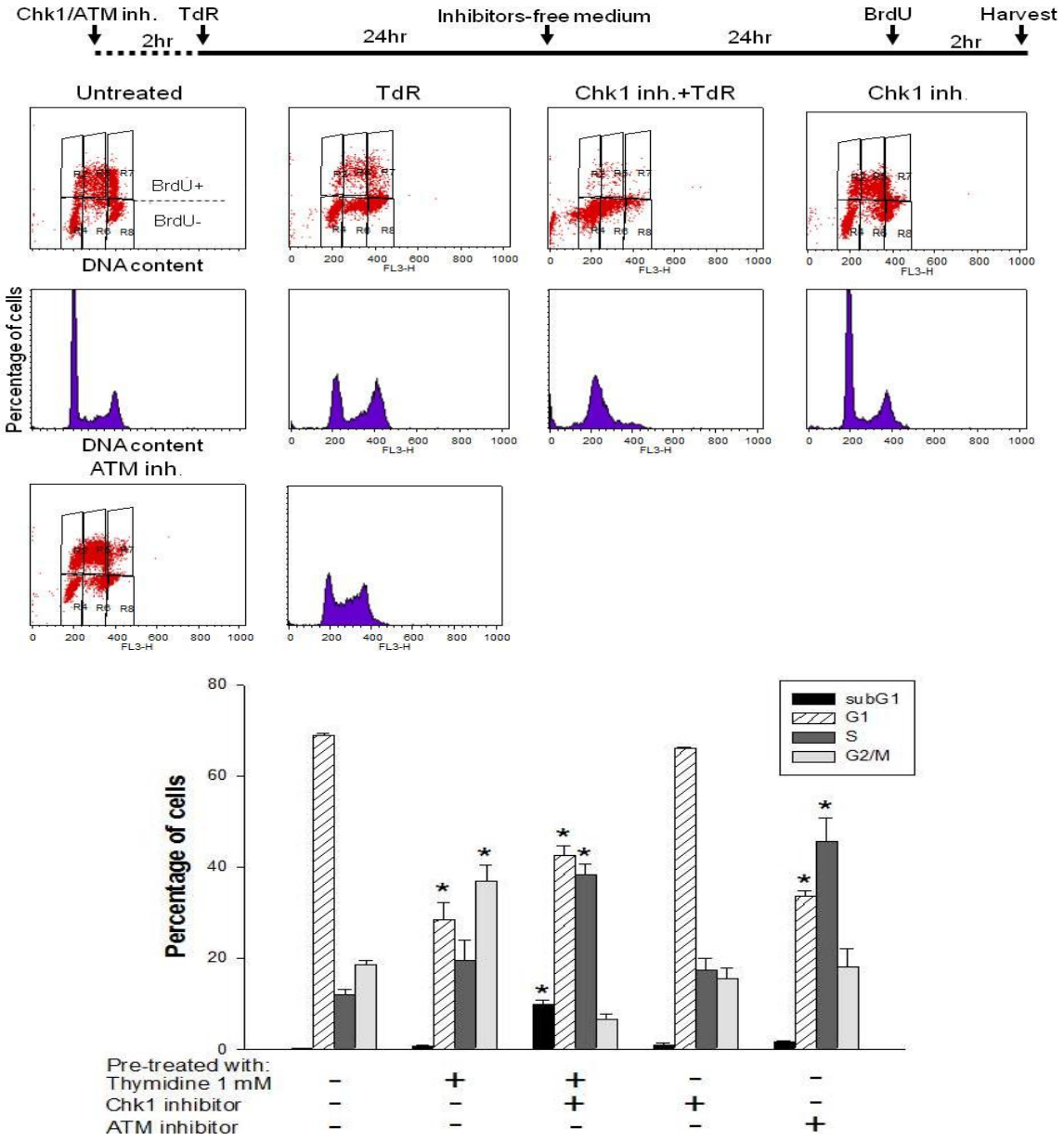



**FIGURE5.12. DNA replication was resumed after released from inhibition.** hTERT-NHU cells were treated with the 1mM thymidine with or without CHK1 inhibitor Gö6976 (1μl) or ATM inhibitor KU55933 (10μM) for 24h before removal of the inhibitor and transfer to inhibitor-free medium for a further 24h. The treated cells along with untreated control cells (grown in inhibitor-free medium for the full 48h) were labelled with BrdU (10μM) for 2h before harvest and analysis of DNA content and BrdU incorporation by flow cytometry. The bar graph presents the distributions of the cells in the different phases of the cell cycle. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

## 5.3.8 The potential interaction between ATM inhibition and autophagocytosis.

Intriguingly, there was an autophagy-like (bubbles and granules formation) morphological changes shown within ATM inhibitor-incubated hTERT-NHU cells after a 2hr treatment but not in other treated groups (Fig. 5.12A).

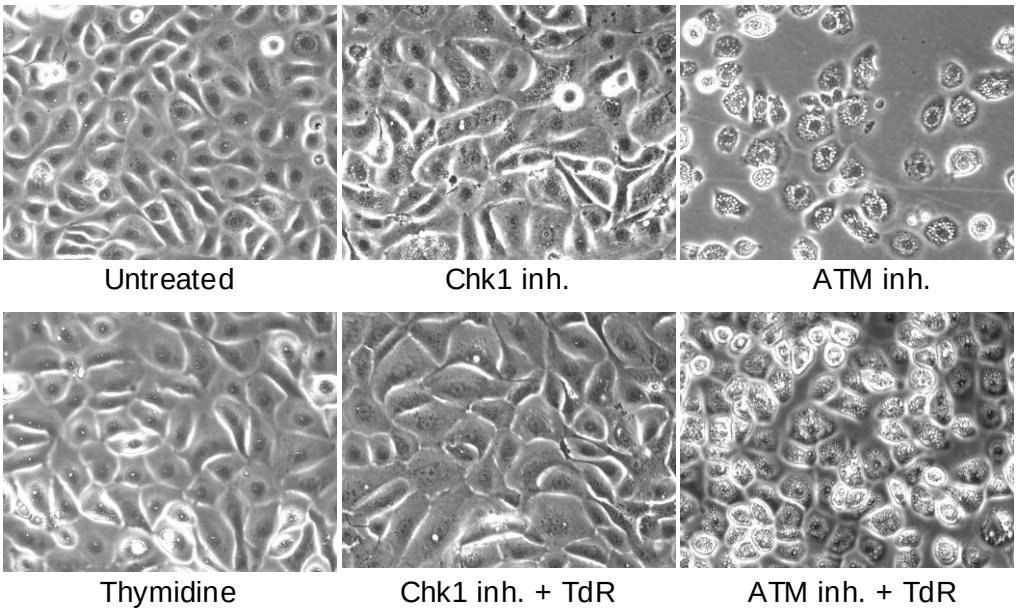



**FIGURE5.13A. ATM inhibitor treatment resulted in autophagy-like morphological alteration.** hTERT-NHU cells were treated with the 1mM thymidine with or without CHK1 inhibitor Gö6976 (1μl) or ATM inhibitor KU55933 (10μM) for 24h and observed by 20X object lens of microscope directly.

Autophagy is a mechanism that cell degrades its unnecessary or dysfunctional cellular organs by lysosomes to maintain cellular energy levels for cell survival in response to starvation or stresses. Recently, ATM activation was reported to be necessary in ROS-induced autophagy in MCF7 cells (Alexander et al.,

2010a; Alexander et al., 2010b) and also required for self-renewal of haematopoietic stem cells (Ito et al., 2004). In the past decade, it has been published that the rat microtubule-associated protein 1 light chain 3 (LC3) is associated to the autophagosome membranes formation and essential for autophagy. During the process of autophagy, the cytosolic LC3-I was converted to membrane bound LC3-II (Kabeya et al., 2000). Therefore the increasing level of LC3-II was widely accepted as a powerful marker of autophagy.

To examine whether this ATM suppression-induced morphological changes resulted from autophagy and associated with ROS, the alteration of LC3 protein in response to ATM inhibition with or without the ROS inhibitor NAC were tested. However in this case, although the level of LC3-II increased dramatically, no evidence of the decrease of LC3-1 was shown in response to KU55933 caused ATM inhibition (Fig. 5.12B). Furthermore, this ATM inhibitor treatment-induced morphological aberration also could not be rescued by pre-incubation of the ROS inhibitor NAC (Fig. 5.12C).

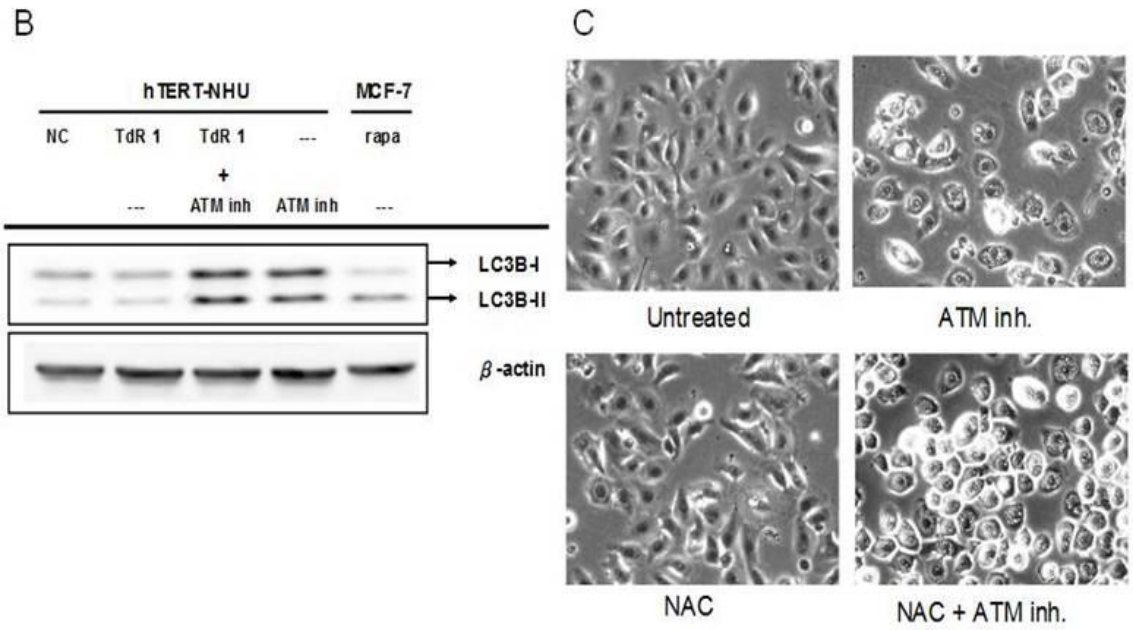
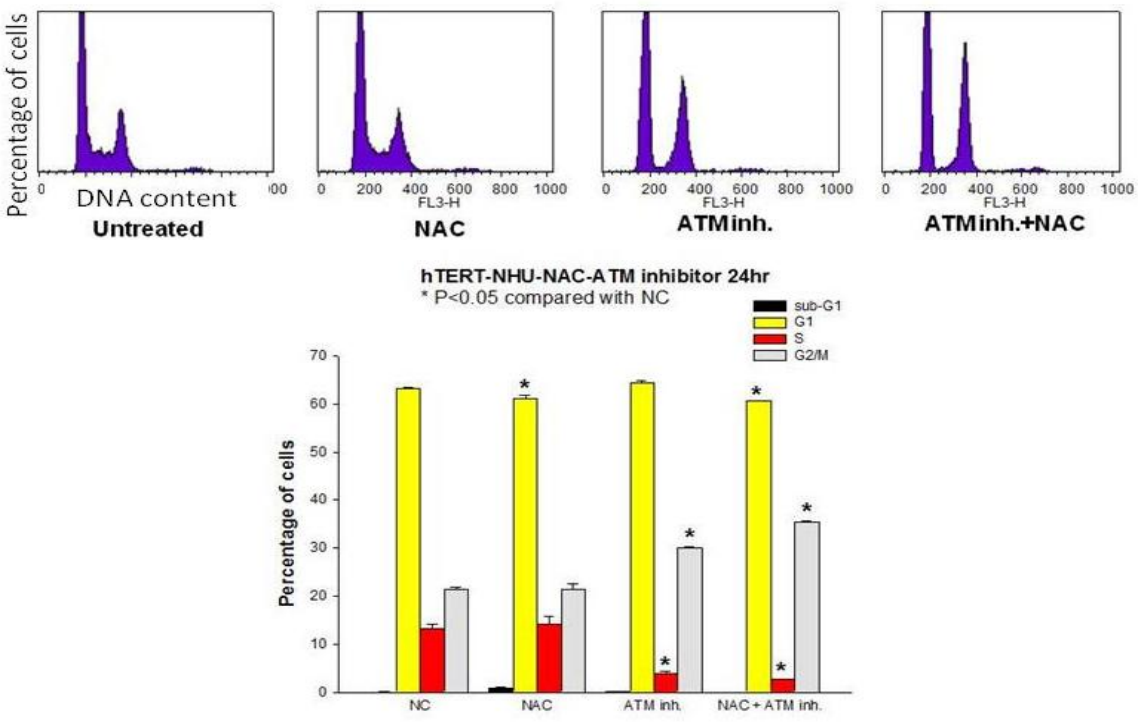



**FIGURE 5.12B, C. ATM inhibitor treatment-induced morphological alteration is independent with autophagy or reactive oxygen species (ROS).** (B) hTERT-NHU cells treated with the 1mM thymidine with or without ATM inhibitor KU55933 (10μM) for 24h were harvested and analysed for the marker of autophagy, LC3 by Western blotting. 100 uM 5hr repamycin-treated MCF-7 served as positive control (Klionsky et al., 2008; Klionsky et al., 2007). β-actin levels are presented as loading controls. (C) hTERT-NHU cells treated with the ATM inhibitor KU55933 (10μM) for 24h with or without the ROS inhibitor NAC (100μM) pre-incubation were observed by 20X object lens of microscope directly.




The cell cycle analysis and bar graph presents the distributions of the cells in the different phases of the cell cycle. Values presented represent the mean of at least three independent experiments ±standard deviation. * P<0.05 compared with untreated negative control (NC) cells.

Altogether, ATM inhibition-induced morphological changes in hTERT-NHU cells seem to be independent of autophagy or reactive oxygen species. Unfortunately, where those cellular granules and bubbles come from is still a mystery. The details of how ATM might participate in epithelial cell re-programming need more studies.

## 5.4 Discussion

ATM has previously been reported to play a novel role in response to DNA replication stress in the absence of DSBs (Bolderson et al., 2004). Here the data further confirm that ATM is activated as an early regulator in hTERT-NHU cells in response to thymidine caused replication stress. While ATM activation has widely been recognised as the sensor of DSB formation, other pathways that repair stalled replication forks could generate the transient double-strand ends that activate ATM. For instance stalled forks in such cells under replication stress may be more likely to regress and generate a “chicken foot structure” which forms transient DNA ends or breaks during the resolution of stalled forks could potentially trigger ATM activation (Courcelle et al., 2003; Petermann and Helleday, 2010).

Intriguingly, in addition to the role as an early regulator following DNA replication stress in hTERT-NHU cells, ATM inhibition is also demonstrated to triggers a $G_1$ checkpoint that controls S-phase entry. This answers the question of why no Ser4/8 phosphorylation was detected following treated ATM inhibitor under these conditions and potentially indicates that functional ATM signalling transduction pathway was crucially responsible for this

phosphorylation. However the deficiency in Ser4/8 RPA2 phosphorylation may simply be due to a failure of cells treated with the ATM inhibitor to enter S-phase. Although the role of ATM in the activation of the $G_1$-S checkpoint in response to DNA double-strand breaks has been well studied, the requirement for ATM in the $G_1$-S transition in unstressed cells is still poorly understood.

Notably ATM was found to play a role in regulating oxidative stress, which is necessary for the self-renewal of mouse haematopoietic stem cells (Block et al., 2004). $p16^{INK4a}$ is activated by an accumulation of reactive oxygen species (ROS) in cells derived from $ATM^{-/-}$ mice and in turn inhibits RB phosphorylation which leads to the block of S-phase entry. In hTERT-NHU cells although RB phosphorylation was reduced after treatment with the ATM inhibitor, there was no $p16^{INK4a}$ accumulation detected and the ROS inhibitor NAC did not reverse the $G_1$ block. In contrast, the levels of the CDK4/6 inhibitor $p19^{INK4D}$ increased accompanied reduced levels of cyclin D1 and CDK4 under these conditions. These alterations are consistent with the $G_1$ checkpoint activation that restricts S-phase entry. Why ATM is required for the $G_1$/S-phase transition needs further studies. In 2005, the induction of $p19^{INK4D}$ in response to ultraviolet light in neuroblastoma cells was reported to enhance UV-induced repair and protect

cells from apoptosis (Ceruti et al., 2005), this may point out a possibility that ATM protects these cells from some sort of ROS independent endogenous DNA damages. However, since $p19^{INK4D}$ can also be induced by the hormonal form of vitamin D3 and retinoids leading to cell cycle arrest (Tavera-Mendoza et al., 2006), the induction of $p19^{INK4D}$ may simply due to that ATM participates in regulation of growth signal transduction pathway for the bladder epithelial cells. In contrast to hTERT-NHU cells, the ATM requirement for S-phase entry was not shown in bladder cancer cell lines as these cells accumulated in S-phase following treated with the ATM inhibitor and committed to apoptosis in response to the combination of ATM and replication inhibitors. Thus this may be a consequence of disruptions of the $G_1$/S checkpoint caused by defects of the RB pathway that are commonly found in tumourigenesis (Di Fiore et al., 2013) or just because the novel role for ATM has lost during the development of bladder cancers.

Replication stress or DNA damage is increased during oncogenic transformation, which makes them more dependent upon DNA damage response pathways for cell survival (Bartkova et al., 2006; Bester et al., 2011; Vafa et al., 2002) . To cope with this stress many tumour cell lines elevate

levels of CHK1 and show dependence on it for protection from apoptosis under DNA replication stress (Verlinden et al., 2007). In addition to Chk1, this study finds that some highly malignant bladder tumour cell lines also showed dependence upon ATM to protect them from apoptosis. Although a combination of ATM and replication inhibitors may provide a novel route to selectively kill bladder cancer cells with no damage on normal urothelial cells, there is no doubt that further studies will be required to examine which type of tumour cells are more favourable in response to such therapies. Taken together, ATM/Chk1 may be an attractive target for clinical anti-cancer therapy as DNA replication stress-induced cell death is specific to ATM/Chk1 suppressed tumour cells but not normal urothelial cells. The most important of all, this therapeutic is reversible in normal non-tumour cells.

# Chapter 6 Discussion

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## 6.1 Introduction

Chk1 plays a critical role in cell cycle progression in response to DNA damage or even in undisturbed cells. In the past decade, the function of Chk1 in response to DNA replication stress has been well established in tumour cells or normal fibroblasts. DNA replication stress has been demonstrated to be cytotoxic to Chk1 depleted tumour cells. Furthermore, ATM was previously reported to play a role in Chk1 regulation. However, the effect of Chk1 inhibition and the correlation between ATM and Chk1 in “normal” cells are still not clearly understood. In this study, the novel roles of Chk1 and ATM are shown in normal urothelial cells.

### 6.1.1 Loss of CHK1 or ATM results in cell death in response to DNA replication stress in tumour cells but not in normal urothelial cells.

Consistent with many other studies, Chk1 inhibition by Gö6976 in EJ bladder cancer cells led to serious cell death indicates that tumour cell survival may be dependent on ATR-Chk1 particularly following DNA replication stress (Meuth, 2010; Myers et al., 2009). However, this dependence was not found in primary

or hTERT immortalized epithelial-like NHU cells. While EJ or TCC-sup bladder cancer cell lines showed evident death response, Chk1 inhibition in hTERT-NHU cells only slows S-phase progression in response to thymidine treatment-induced DNA replication stress, but does not trigger cell apoptosis

As described previously in the chapter of introduction, Chk1 serves as a work horse even in undisturbed cells for cell cycle progression. However in many malignant tumour cells Chk1 has been demonstrated to play a role in protecting cells from death in response to DNA replication stress. Hence it is not surprising that DNA replication inhibitors thymidine or gemcitabine triggered serious cell apoptosis in Chk1 inhibited TCC-sup and even more robust death response in EJ bladder tumour cells. In contrast, normal urothelial cells do not appear to exhibit a similar dependence. Neither totally primary NHU nor human telomerase reverse transcriptase (hTERT) transfected hTERT-NHU cells show cell death in response to this similar treatment.

Furthermore, ATM is found to protect EJ cells as well as Chk1 from replication stress-induced cell apoptosis. Unlike hTERT-NHU cells, EJ fully activates classical DNA damage response pathways with both Ser345 Chk1 and

Ser1981 ATM phosphorylation. When Chk1 kinase activity is suppressed by Chk1 inhibitor Gö6976, the Ser1981 phosphorylated ATM undergoes a dramatic increase in response to genotoxic stress in EJ cells. This is consistent with the report that Chk1 inhibition activates an ATM and DNA-dependent protein kinase (DNAPK)-mediated double strand break response under replication defects. Checkpoint abrogated cells result in ATM and DNA-PK, but not ATR mediated γ-H2AX induction and lethally premature mitosis entry following gemcitabine treatment (McNeely et al., 2010). Thus a DNA replication stress agent like gemcitabine seems to be able to selectively kill highly malignant but not normal urothelial cells after CHK1 or ATM inhibition.

### 6.1.2 CHK1 Ser345 is not the major regulator in normal urothelial cells under DNA replication stress.

It was also found that ATM, but not ATR plays a more important role in hTERT-NHU cells. ATM serves as an early checkpoint in this cell model in response to DNA replication stress and a key regulator of S-phase entry. Although some other groups reported that ATR activation and some ATR downstream proteins can be regulated by ATM. Both ATM (pSer-1981) and Chk1 (pSer-345), but neither ATR (pSer-428) nor Chk2 (pThr-68) are activated in mouse zygotes fertilized with $H_2O_2$-treated sperm to trigger cell cycle arrest to provide time for sperm-derived DNA-damage repair (Wang et al., 2013). ATM has also been found able to regulate a direct ATR activator TopBP1 by phosphorylation on Ser-1131 in Xenopus egg extracts. A mutation that restricted phosphorylation at TopBP1 at this site, produced a defective ATR-dependent activation of Chk1 in response to DNA damage (Yoo et al., 2007). However this ATM-mediated ATR activation does not appear to be responsible for thymidine-induced DNA replication stress in hTERT-NHU cells. Since Chk1 Ser345 phosphorylation site is well known to be a key downstream regulator in response to activated ATR, it is not detected in hTERT-NHU cells followed by thymidine treatment. Additionally, there is no

RPA Ser33 phosphorylation, the major phosphorylation site of ATR, but Ser4/8 phosphorylation of RPA2 can be observed following thymidine treatment. These data suggest that the ATR-Chk1 pathway does not respond in these cells and that ATM or DNAPK signalling cascades may play the initial response to DNA replication stress in normal urothelial cells.

### 6.1.3 The role of RPA hyper-phosphorylation in DNA replication stress.

In HCT 116 colon tumour cells, RPA hyper-phosphorylation after thymidine-induced DNA replication stress was found in Chk1 inhibited cells. However this hyper-phosphorylated form of RPA was not apparently induced in hTERT-NHU cells even treated with both thymidine and Chk1 inhibitor. Unfortunately, so far the role of RPA hyper-phosphorylation is still not clearly understood. Two possible mechanisms of RPA hyper-phosphorylation were previously hypothesized (Zou et al., 2006). First, the hyper-phosphorylation of RPA may serve the recognition by hyper-phosphorylation binding proteins. It is possible that the hyper-phosphorylation of RPA is required for regulation of proteins such as checkpoints in response to different genetic stresses. Second, RPA hyper-phosphorylation results in protein structure transformation and leads to the disruption of RPA-DNA and RPA-protein interactions (Zou et al., 2006). It is possible that the checkpoint-induced RPA hyper-phosphorylation may down regulate the DNA replication initiation and also suppress recombination as hyper-phosphorylation mimicking RPA is not able to associate with replication centres in vivo (Vassin et al., 2004).

### 6.1.4 The potential interaction between ATM and RPA phosphorylation

In this study, it has been found that ATM acts an early regulator rather than ATR-Chk1 in response to thymidine treatment-induced DNA replication stress in hTERT-NHU cells. Although RPA has already been reported to coat ssDNA and recruit ATR to trigger ATR-CHK1 checkpoint signalling pathway during replication stress (Zou and Elledge, 2003), it remains unclear that whether RPA is also involved in ATM-dependent checkpoint cascades. Here I showed that the ATM inhibitor is able to suppress RPA Ser4/8 phosphorylation directly under these conditions, suggesting that the ATM signalling pathway may participate in this phosphorylation although the deficiency of RPA Ser4/8 phosphorylation is most likely due to the failure such cells to enter S-phase.

### 6.1.5 The expression of γ-H2AX in combined treatment of DNA replication stress and CHK1 inhibitor.

In addition, I also found that γ-H2AX is mainly expressed as well as Ser4/8 hyper-phosphorylated RPA and p-p53 (S15) in thymidine treated hTERT-NHU cells following Chk1 inhibition by the inhibitor Gö6976. It is consistent with the fact that RPA is competent to associate with DNA damage foci and co-localize with γ-H2AX under DNA damage or DNA replication stress (Vassin et al., 2004).

γ-HA2X is generally considered as a marker of DNA double strand breaks (DSB). Following DSB, the histone subunit H2AX was rapidly (in 30 minutes) phosphorylated at c-terminus by one or more DSB-activated PI3K-like kinases including ATM, ATR and DNA-PK to form γ-H2AX at near DSB sites (Bonner et al., 2008; Rogakou et al., 1999). The growing γ-H2AX focus may serve to open up the chromatin structure and result in the accumulation of many checkpoint and DNA repair-associated proteins to promote DNA damage signalling pathway and DNA repair (Kruhlak et al., 2006). However, as thymidine does not induce DNA damage or DNA double strand breaks (Bolderson et al., 2004), but delays the DNA replication folk progression, it is unclear why thymidine

treatment still caused γ-H2AX expression in the hTERT-NHU cells (Gagou et al., 2010). In addition to DSB, although H2AX is reported to be phosphorylated by ultraviolet C-activated ATR kinase activity in response to DNA single-stranded regions (Stiff et al., 2006), it has been demonstrated that ATR is not the major regulator in hTERT-NHU cells under DNA replication stress. Whether its expression is associated with stalled replication-induced folk regression and ATM activation needs further studies.

### 6.1.6 The novel role of ATM in cell cycle S-phase entry in hTERT-NHU cells.

In my study, ATM is also found to be activated as an early event in response to thymidine-induced DNA replication stress. ATM has been reported as a key sensor in response to DNA double strand breaks (Lavin, 2007; Lavin and Kozlov, 2007; Niida and Nakanishi, 2006) or DNA ends (Bolderson et al., 2004). It is surprising to see that ATM inhibitor activation results in a $G_1$ phase block that was revealed by a lack of S phase entry. Interestingly, autophagy-like morphological changes were observed, but other autophagy-like features were missing. Notably, ATM is reported to be necessary for the self-renewal of mouse haematopoietic stem cells by regulating oxidative stress (Block et al., 2004). Whether the defect of cycle $G_1$-S phase progression in normal urothelial cells results in some kind of cell re-programming needs further examination.

### 6.1.7 Limitations

In this study, I have reported a novel role of CHK1 and ATM in immortalized hTERT-NHU cells. These findings seem to suggest an attractive new target for cancer therapy however there are still many details remain unclear. How the mechanism of Chk1 works, how ATM deficiency prevents $G_1$/S phase transition in normal urothelial cells, why only Chk1 inhibited tumour cells take effect following replication stress are still poorly understood. Can this death response also apply to other bladder tumour cells in addition to EJ and TCC-sup? I have tried similar treatments on lower grade tumour cells such as RT4 or RT112, however less evidence of cell apoptosis was shown on those cell types. The cell death response is much lower than in more malignant Tcc-sup and EJ cells. In addition, I have found that activation of ATR-Chk1 signalling cascades is deficient in response to DNA replication stress in hTERT-NHU cells by many clues. However I have not analysed yet ATR activation.

The most important of all, transformed immortalized NHU cells are not totally normal ones. Unfortunately, primary normal human urothelial (NHU) cells were difficult to analyse. Unlike cancer cell lines, even immortalized hTERT-NHU

cells are really difficult to work with. Re-feed with the specific and expensive KSFM culture medium is needed three times per week. Additionally, they grow slowly and are unstable. Attempts to culture them in petri-dish for a long-term treatment have never seen successful. Furthermore, the Chk1 inhibitor Gö6976 is not extremely specific to Chk1 kinase, hence the Chk1 siRNA may be a good comparison to the inhibitor. I did attempt to deplete Chk1 with siRNAs but these experiments were unsuccessful. Generally groups here have not been successful with siRNA or shRNA mediated knockdowns of target genes in the hTERT-NHU cells.

### 6.1.8 Future work

In addition to cell cycle checkpoint signalling, mitogen-activated protein kinase (MAPK) cascades are critical signalling pathways and well-known proto-oncogenes which regulate cell proliferation, survival and differentiation of both normal and malignant cells (Roberts and Der, 2007). Hence, it is not surprising that aberrant regulation of MAPK cascades contribute to cancer and other human diseases. Interestingly, exposure of various multiple myeloma cells to non-cytotoxic concentrations of Chk1 inhibitors induced DNA damage and a little cell death accompanied by Ras and ERK1/2 activation (Dai et al., 2008; Dai et al., 2001). Furthermore, interruption of the MAPK signalling cascade also enhanced Chk1 inhibitor-induced DNA damage and apoptosis both *in vitro* and *in vivo* (Dai et al., 2008). Deregulated Ras signalling was found to compromise DNA damage checkpoint recovery after completing DNA repair in *S. cerevisiae* yeast. This indicated a new role for the Ras signalling pathway as a regulator of DNA damage checkpoint recovery (Wood and Sanchez, 2010). At the same time, the ATR-Chk1 pathway was shown to be able to limit oncogenic Ras transformation induced genomic instability. In cultured murine embryonic fibroblasts (MEFs), the observation of elevated genomic instability and synergistically increased lethality were found due to the

combination of ATR suppression and oncogenic Ras activation (Gilad et al., 2010). However whether Chk1 suppression also affects MAPK pathway is still not clear. Together these studies, Ras/MEK/ERK signalling cascade may represent an interaction or functional compensation with Chk1 checkpoint signalling pathway. It would be interesting to examine the potential interaction between MAPK and ATM-Chk1 particularly in normal epithelial cells.

In addition, to further examine whether the cell survival of hTERT-NHU cells in response to CHK1 inhibitor under DNA replication stress is ATR dependent or not, ATR inhibitor might be an interesting treatment especially combined with CHK1 inhibitor.

Furthermore, DNA replication stress resulting from oncogene expression was shown to lead to genome instability in early cancer stage (Di Micco et al., 2006). Overexpression of oncogenes such as HPV-16 E6/E7 and cyclin E has been demonstrated to lead to abnormal RB-E2F pathway which resulted in insufficient nucleotide pools during DNA replication and finally caused DNA damage and genome instability (Bester et al., 2011). Since the thymidine treatment in current step of this study also caused nucleotide pool unbalance

and deficiency and which was shown to be connected to checkpoint kinase activation. Hence, it would be interesting to make stable cell lines with different kind of oncogenes such as cyclin E, c-myc for mimicking the condition and investigate the potential role of checkpoint mechanism in early oncogenesis stages in normal epithelial cells.

Additionally, directly examining the status of DNA strand in response to loss of ATM-Chk1 under DNA replication stress would be helpful to understand the detailed mechanism of how this system works and try to answer why only malignant tumour cells are selectively killed.

### 6.1.9 Conclusion

Cancer is considered as one of the greatest death threats to human beings nowadays. Unfortunately there are still many difficulties on the way for beating cancer. Several newer types of treatment such as targeted therapy are now being studied. Many targeted drugs including sunitinib (Sutent®), lapatinib (Tykerb®), erlotinib (Tarceva®), trastuzumab (Herceptin®), and gefitinib (Iressa®) are already being used to treat different types of cancer. However the effect of these drugs on bladder tumours is not as good as on their original targets. In comparison to other solid tumors such as lung, breast and prostate, no targeted therapies for bladder cancer have brought to the clinic and there is no new agent being registered in the past two decades (Dovedi and Davies, 2009). Recently, although some groups have also declared they find some new targets for targeted therapeutics, their treatments usually result in some side effects or irreversible cell senescence on normal cells. This work identified a huge different consequence of ATM/CHK1 inhibition between malignant and normal cells that the death response is specific to advanced cancer cells but not non-tumour urothelial cells under DNA replication stress. Whereas kinase activity-suppressed Chk1 only slowed S phase progression, ATM inhibition triggered p19 activation and down-regulated cyclin D1, CDK4 and p-RB in

hTERT-NHU cells (Fig. 6). These findings suggest that ATM and CHK1 signalling cascades may be an attractive target for cancer therapy in the future.

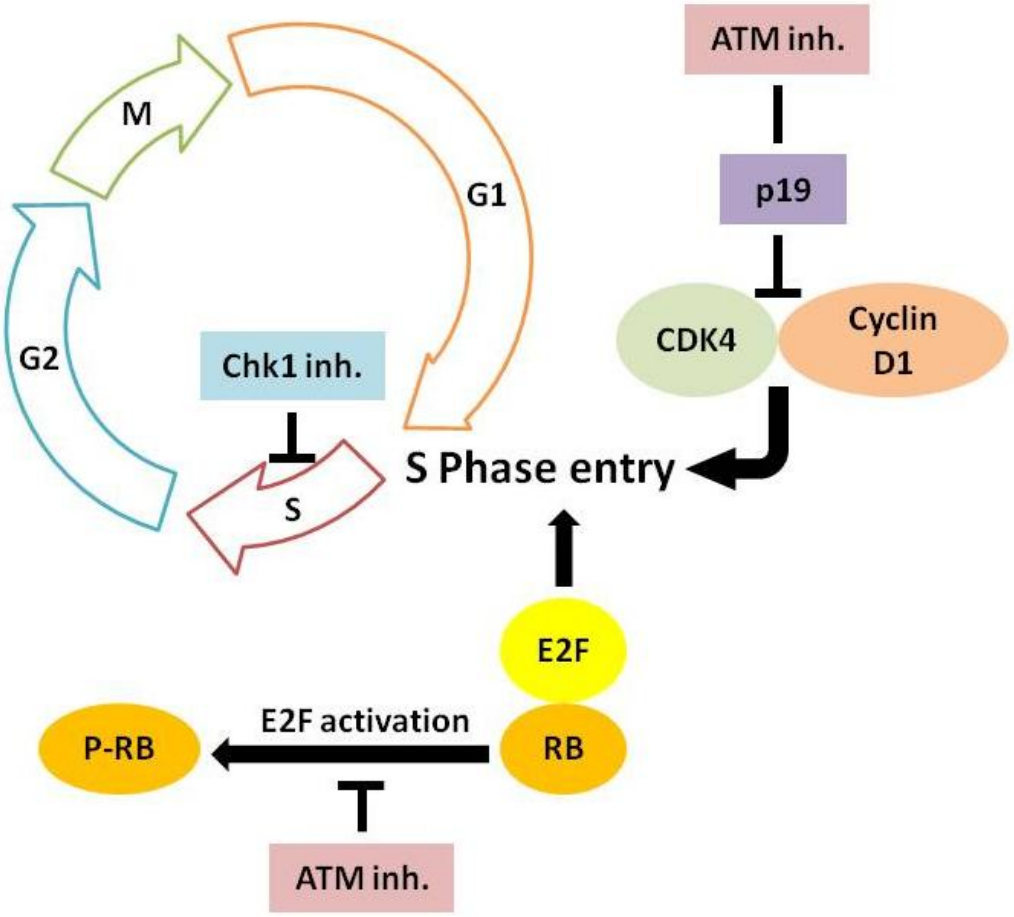


**FIGURE6. Schematic representation of the different signalling transduction pathways in response to Chk1/ATM inhibitor in hTERT-NHU cells.** Whereas kinase activity-suppressed Chk1 only slowed S phase progression, ATM inhibition resulted in p19 activation and further down-regulated cyclin D1, CDK4 and p-RB, which triggered $G_1$ checkpoint activation and prevented S phase entry in hTERT-NHU cells.

# Chapter 7 References

Aaltonen, V., Koivunen, J., Laato, M., and Peltonen, J. (2007). PKC inhibitor Go6976 induces mitosis and enhances doxorubicin-paclitaxel cytotoxicity in urinary bladder carcinoma cells. Cancer letters *253*, 97-107.

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes & Development *15*, 2177-2196.

Abraham, R.T. (2004). PI 3-kinase related kinases: [`]big' players in stress-induced signaling pathways. DNA Repair *3*, 883-887.

Adams, J.M., and Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene *26*, 1324-1337.

Akinaga, S., Nomura, K., Gomi, K., and Okabe, M. (1993). Enhancement of antitumor activity of mitomycin C in vitro and in vivo by UCN-01, a selective inhibitor of protein kinase C. Cancer Chemother Pharmacol *32*, 183-189.

Alexander, A., Cai, S.-L., Kim, J., Nanez, A., Sahin, M., MacLean, K.H., Inoki, K., Guan, K.-L., Shen, J., Person, M.D., *et al.* (2010a). ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proceedings of the National Academy of Sciences *107*, 4153-4158.

Alexander, A., Kim, J., and Walker, C.L. (2010b). ATM engages the TSC2/mTORC1 signaling node to regulate autophagy. Autophagy *6*, 672-673.

Amadori, D., Volpi, A., Maltoni, R., Nanni, O., Amaducci, L., Amadori, A., Giunchi, D.C., Vio, A., Saragoni, A., and Silvestrini, R. (1997). Cell proliferation as a predictor of response to chemotherapy in metastatic breast cancer: a prospective study. Breast Cancer Res Treat *43*, 7-14.

Anantha, R.W., Sokolova, E., and Borowiec, J.A. (2008). RPA phosphorylation facilitates mitotic exit in response to mitotic DNA damage. Proc Natl Acad Sci U S A *105*, 12903-12908.

Anantha, R.W., Vassin, V.M., and Borowiec, J.A. (2007). Sequential and synergistic modification of human RPA stimulates chromosomal DNA repair. J Biol Chem *282*, 35910-35923.

Ashwell, S., Janetka, J.W., and Zabludoff, S. (2008). Keeping checkpoint kinases in line: new selective inhibitors in clinical trials. Expert opinion on investigational drugs *17*, 1331-1340.

Baldin, V., Lukas, J., Marcote, M.J., Pagano, M., and Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev *7*, 812-821.

Ball, H.L., Myers, J.S., and Cortez, D. (2005). ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation. Molecular Biology of the Cell *16*, 2372-2381.

Balter, M., and Vogel, G. (2001). Nobel prize in physiology or medicine. Cycling toward Stockholm. Science *294*, 502-503.

Banin, S., Moyal L Fau - Shieh, S., Shieh S Fau - Taya, Y., Taya Y Fau - Anderson, C.W., Anderson Cw Fau - Chessa, L., Chessa L Fau - Smorodinsky, N.I., Smorodinsky Ni Fau - Prives, C., Prives C Fau - Reiss, Y., Reiss Y Fau - Shiloh, Y., Shiloh Y Fau - Ziv, Y., *et al.* (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage.

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D., *et al.* (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell *86*, 159-171.

Bartek, J., and Lukas, J. (2001). Mammalian G1- and S-phase checkpoints in response to DNA damage. Current Opinion in Cell Biology *13*, 738-747.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell *3*, 421-429.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., *et al.* (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

Behrens, M.M., Strasser, U., and Choi, D.W. (1999). Go 6976 is a potent inhibitor of neurotrophin-receptor intrinsic tyrosine kinase. Journal of neurochemistry *72*, 919-924.

Bennett, C., Tomlinson, C., Michalowski, A., Chu, I., Luger, D., Mittereder, L., Aprelikova, O., Shou, J., Piwinica-Worms, H., Caplen, N., *et al.* (2012). Cross-species genomic and functional analyses identify a combination therapy using a CHK1 inhibitor and a ribonucleotide reductase inhibitor to treat triple-negative breast cancer. Breast Cancer Research *14*, R109.

Bester, A., Roniger, M., Oren, Y., Im, M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D., and Kerem, B. (2011). Nucleotide Deficiency Promotes Genomic Instability in Early Stages of Cancer Development. Cell *145*, 435-446.

Blackwood, E., Epler, J., Yen, I., Flagella, M., O'Brien, T., Evangelista, M., Schmidt, S., Xiao, Y., Choi, J., Kowanetz, K., *et al.* (2013). Combination Drug Scheduling Defines a ? indow of Opportunity??for Chemopotentiation of Gemcitabine by an Orally Bioavailable, Selective ChK1 Inhibitor, GNE-900. Molecular Cancer Therapeutics *12*, 1968-1980.

Block, W.D., Yu, Y.P., and Lees-Miller, S.P. (2004). Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases (PIKKs) are required for DNA damage-induced phosphorylation of the 32 kDa subunit of replication protein A at threonine 21. Nucleic Acids Research *32*, 997-1005.

Bolderson, E., Scorah, J., Helleday, T., Smythe, C., and Meuth, M. (2004). ATM is required for the cellular response to thymidine induced replication fork stress. Human Molecular Genetics *13*, 2937-2945.

Bonner, W.M., Redon, C.E., Dickey, J.S., Nakamura, A.J., Sedelnikova, O.A., Solier, S., and Pommier, Y. (2008). [gamma]H2AX and cancer. Nat Rev Cancer *8*, 957-967.

Bric, A., Miething, C., Bialucha, C.U., Scuoppo, C., Zender, L., Krasnitz, A., Xuan, Z., Zuber, J., Wigler, M., Hicks, J., *et al.* (2009). Functional Identification of Tumor-Suppressor Genes through an In Vivo RNA Interference Screen in a Mouse Lymphoma Model. Cancer Cell *16*, 324-335.

Brill, S.J., and Stillman, B. (1991). REPLICATION FACTOR-A FROM SACCHAROMYCES-CEREVISIAE IS ENCODED BY 3 ESSENTIAL GENES COORDINATELY EXPRESSED AT S-PHASE. Genes & Development *5*, 1589-1600.

Brush, G.S., Kelly, T.J., and Stillman, B. (1995). Identification of eukaryotic DNA replication proteins using simian virus 40 in vitro replication system. In DNA Replication (San Diego: Academic Press Inc), pp. 522-548.

Bubenik, J., Baresova, M., Viklicky, V., Jakoubkova, J., Sainerova, H., and Donner, J. (1973). Established cell line of urinary bladder carcinoma (T24) containing tumour-specific antigen. International journal of cancer Journal international du cancer *11*, 765-773.

Bunch, R.T., and Eastman, A. (1996). Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G2-checkpoint inhibitor. Clin Cancer Res *2*, 791-797.

Busby, E.C., Leistritz, D.F., Abraham, R.T., Karnitz, L.M., and Sarkaria, J.N. (2000). The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. Cancer Res *60*, 2108-2112.

Byun, T.S., Pacek, M., Yee, M.-c., Walter, J.C., and Cimprich, K.A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes & Development *19*, 1040-1052.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Carlessi, L., De Filippis, L., Lecis, D., Vescovi, A., and Delia, D. (2009). DNA-damage response, survival and differentiation in vitro of a human neural stem cell line in relation to ATM expression. Cell Death Differ *16*, 795-806.

Carrassa, L., and Damia, G. (2011). Unleashing Chk1 in cancer therapy. Cell Cycle *10*, 2121-2128.

Catto, J.W.F., Miah, S., Owen, H.C., Bryant, H., Myers, K., Dudziec, E., Larr 瞹, S., Milo, M., Rehman, I., Rosario, D.J., *et al.* (2009). Distinct MicroRNA Alterations Characterize High- and Low-Grade Bladder Cancer. Cancer Research *69*, 8472-8481.

Cerqueira, N.M., Fernandes, P.A., and Ramos, M.J. (2007). Understanding ribonucleotide reductase inactivation by gemcitabine. Chemistry (Weinheim an der Bergstrasse, Germany) *13*, 8507-8515.

Ceruti, J.M., Scassa, M.E., Flo, J.M., Varone, C.L., and Canepa, E.T. (2005). Induction of p19INK4d in response to ultraviolet light improves DNA repair and confers resistance to apoptosis in neuroblastoma cells. Oncogene *24*, 4065-4080.

Chen, C., and Kolodner, R.D. (1999). Gross chromosomal rearrangements in Saccharomyces cerevisiae replication and recombination defective mutants. Nature genetics *23*, 81-85.

Chen, C., Umezu, K., and Kolodner, R.D. (1998). Chromosomal rearrangements occur in S. cerevisiae rfa1 mutator mutants due to mutagenic lesions processed by double-strand-break repair. Mol Cell *2*, 9-22.

Cho, S.H., Toouli, C.D., Fujii, G.H., Crain, C., and Parry, D. (2005). Chk1 is essential for tumor cell viability following activation of the replication checkpoint. Cell Cycle *4*, 131-139.

Ciccia, A., and Elledge, S.J. (2010). The DNA Damage Response: Making It Safe to Play with Knives. Molecular Cell *40*, 179-204.

Clarke, C.A., and Clarke, P.R. (2005). DNA-dependent phosphorylation of Chk1 and Claspin in a human cell-free system. The Biochemical journal *388*, 705-712.

Cliby, W.A., Roberts, C.J., Cimprich, K.A., Stringer, C.M., Lamb, J.R., Schreiber, S.L., and Friend, S.H. (1998). Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. Embo j *17*, 159-169.

Cole, K.A., Huggins, J., Laquaglia, M., Hulderman, C.E., Russell, M.R., Bosse, K., Diskin, S.J., Attiyeh, E.F., Sennett, R., Norris, G., *et al.* (2011). RNAi screen of the protein kinome identifies checkpoint kinase 1 (CHK1) as a therapeutic target in neuroblastoma. Proceedings of the National Academy of Sciences *108*, 3336-3341.

Courcelle, J., Donaldson, J.R., Chow, K.-H., and Courcelle, C.T. (2003). DNA Damage-Induced Replication Fork Regression and Processing in Escherichia coli. Science *299*, 1064-1067.

Curtin, N.J. (2012). DNA repair dysregulation from cancer driver to therapeutic target. Nat Rev Cancer *12*, 801-817.

Dai, Y., Chen, S., Pei, X.-Y., Almenara, J.A., Kramer, L.B., Venditti, C.A., Dent, P., and Grant, S. (2008). Interruption of the Ras/MEK/ERK signaling cascade enhances Chk1 inhibitor-induced DNA damage in vitro and in vivo in human multiple myeloma cells. Blood *112*, 2439-2449.

Dai, Y., Yu, C., Singh, V., Tang, L., Wang, Z., McInistry, R., Dent, P., and Grant, S. (2001). Pharmacological Inhibitors of the Mitogen-activated Protein Kinase (MAPK) Kinase/MAPK Cascade Interact Synergistically with UCN-01 to Induce Mitochondrial Dysfunction and Apoptosis in Human Leukemia Cells. Cancer Research *61*, 5106-5115.

Davies, M.A., and Samuels, Y. (2010). Analysis of the genome to personalize therapy for melanoma. Oncogene *29*, 5545-5555.

Dent, P., Tang, Y., Yacoub, A., Dai, Y., Fisher, P.B., and Grant, S. (2011). CHK1 inhibitors in combination chemotherapy: thinking beyond the cell cycle. Molecular interventions *11*, 133-140.

Di Fiore, R., D'Anneo, A., Tesoriere, G., and Vento, R. (2013). RB1 in cancer: different mechanisms of RB1 inactivation and alterations of pRb pathway in tumorigenesis. J Cell Physiol *228*, 1676-1687.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Giovanni Nuciforo, P., Bensimon, A., *et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Dovedi, S., and Davies, B. (2009). Emerging targeted therapies for bladder cancer: a disease waiting for a drug. Cancer and Metastasis Reviews *28*, 355-367.

Durkin, S.G., Arlt, M.F., Howlett, N.G., and Glover, T.W. (2006). Depletion of CHK1, but not CHK2, induces chromosomal instability and breaks at common fragile sites. Oncogene *25*, 4381-4388.

Ewald, B., Sampath, D., and Plunkett, W. (2007). H2AX phosphorylation marks gemcitabine-induced stalled replication forks and their collapse upon S-phase checkpoint abrogation. Molecular Cancer Therapeutics *6*, 1239-1248.

Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature *410*, 842-847.

Fang, Y., Tsao, C.-C., Goodman, B.K., Furumai, R., Tirado, C.A., Abraham, R.T., and Wang, X.-F. (2004). ATR functions as a gene dosage-dependent tumor suppressor on a mismatch repair-deficient background. EMBO J *23*, 3164-3174.

Ferrís, J., Garcia, J., Berbel, O., and Ortega, J.A. (2013). Constitutional and occupational risk factors associated with bladder cancer. TITLEREVISTA *37*, 513-522.

Fox, R.M., Tripp, E.H., and Tattersall, M.H.N. (1980). Mechanism of Deoxycytidine Rescue of Thymidine Toxicity in Human T-Leukemic Lymphocytes. Cancer Research *40*, 1718-1721.

Furnari, B., Rhind, N., and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. Science *277*, 1495-1497.

Gagou, M.E., Zuazua-Villar, P., and Meuth, M. (2010). Enhanced H2AX Phosphorylation, DNA Replication Fork Arrest, and Cell Death in the Absence of Chk1. Molecular Biology of the Cell *21*, 739-752.

Ghebranious, N., and Donehower, L.A. (1998). Mouse models in tumor suppression. Oncogene *17*, 3385-3400.

Gilad, O., Nabet, B.Y., Ragland, R.L., Schoppy, D.W., Smith, K.D., Durham, A.C., and Brown, E.J. (2010). Combining ATR Suppression with Oncogenic Ras Synergistically Increases Genomic Instability, Causing Synthetic Lethality or Tumorigenesis in a Dosage-Dependent Manner. Cancer Research *70*, 9693-9702.

Givalos, N., Gakiopoulou, H., Skliri, M., Bousboukea, K., Konstantinidou, A.E., Korkolopoulou, P., Lelouda, M., Kouraklis, G., Patsouris, E., and Karatzas, G. (2007). Replication protein A is an independent prognostic indicator with potential therapeutic implications in colon cancer. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc *20*, 159-166.

Golding, S.E., Rosenberg, E., Valerie, N., Hussaini, I., Frigerio, M., Cockcroft, X.F., Chong, W.Y., Hummersone, M., Rigoreau, L., Menear, K.A., *et al.* (2009). Improved ATM kinase inhibitor KU-60019 radiosensitizes glioma cells, compromises insulin, AKT and ERK prosurvival signaling, and inhibits migration and invasion. Mol Cancer Ther *8*, 2894-2902.

Goto, H., Izawa, I., Li, P., and Inagaki, M. (2012). Novel regulation of checkpoint kinase 1: Is checkpoint kinase 1 a good candidate for anti-cancer therapy? Cancer Science *103*, 1195-1200.

Grandage, V.L., Everington, T., Linch, D.C., and Khwaja, A. (2006). Go6976 is a potent inhibitor of the JAK 2 and FLT3 tyrosine kinases with significant activity in primary acute myeloid leukaemia cells. British journal of haematology *135*, 303-316.

Graves, P.R., Yu, L., Schwarz, J.K., Gales, J., Sausville, E.A., O'Connor, P.M., and Piwnica-Worms, H. (2000). The Chk1 Protein Kinase and the Cdc25C Regulatory Pathways Are Targets of the Anticancer Agent UCN-01. Journal of Biological Chemistry *275*, 5600-5605.

Green, J., Shibata, M., Yoshidome, K., Liu, M., Jorcyk, C., Anver, M., Wigginton, J., Wiltrout, R., Shibata, E., Kaczmarczyk, S., *et al.* (2000). The C3(1)/SV40 T-antigen transgenic mouse model of mammary cancer: ductal epithelial cell targeting with multistage progression to carcinoma. Oncogene *19*, 1020 - 1027.

Hanahan, D., and Weinberg, R.A. (2000). The Hallmarks of Cancer. Cell *100*, 57-70.
Hanahan, D., and Weinberg, Robert A. (2011). Hallmarks of Cancer: The Next Generation. Cell *144*, 646-674.

Hannon, G.J., and Beach, D. (1994). pl5INK4B is a potentia| effector of TGF-[beta]-induced cell cycle arrest. Nature *371*, 257-261.

Henricksen, L.A., Umbricht, C.B., and Wold, M.S. (1994). RECOMBINANT REPLICATION PROTEIN-A - EXPRESSION, COMPLEX-FORMATION, AND FUNCTIONAL-CHARACTERIZATION. Journal of Biological Chemistry *269*, 11121-11132.

Hickson, I., Zhao, Y., Richardson, C.J., Green, S.J., Martin, N.M.B., Orr, A.I., Reaper, P.M., Jackson, S.P., Curtin, N.J., and Smith, G.C.M. (2004). Identification and Characterization of a Novel and Specific Inhibitor of the Ataxia-Telangiectasia Mutated Kinase ATM. Cancer Research *64*, 9152-9159.

Huang, X., Halicka, H.D., and Darzynkiewicz, Z. (2004). Detection of histone H2AX phosphorylation on Ser-139 as an indicator of DNA damage (DNA double-strand breaks). Current protocols in cytometry / editorial board, J Paul Robinson, managing editor [et al] *Chapter 7*, Unit 7.27.

Huigsloot, M., Tijdens, R.B., and van de Water, B. (2003). Inhibition of protein kinase Calpha enhances anticancer agent-induced loss of anchorage-independent growth regardless of protection against apoptosis by Bcl-2. Molecular pharmacology *64*, 965-973.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N., *et al.* (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature *431*, 997-1002.

Jacobs, B.L., Lee, C.T., and Montie, J.E. (2010). Bladder Cancer in 2010: How Far have We Come? CA: A Cancer Journal for Clinicians *60*, 244-272.

Jia, X.Z., Yang, S.Y., Zhou, J., Li, S.Y., Ni, J.H., An, G.S., and Jia, H.T. (2009). Inhibition of CHK1 kinase by Go6976 converts 8-chloro-adenosine-induced G2/M arrest into S arrest in human myelocytic leukemia K562 cells. Biochem Pharmacol *77*, 770-780.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J *19*, 5720-5728.

Kakehi, Y., Hirao, Y., Kim, W.-J., Ozono, S., Masumori, N., Miyanaga, N., Nasu, Y., and Yokomizo, A. (2010). Bladder Cancer Working Group Report. Japanese Journal of Clinical Oncology *40*, i57-i64.

Kaneko, Y.S., Watanabe, N., Morisaki, H., Akita, H., Fujimoto, A., Tominaga, K., Terasawa, M., Tachibana, A., Ikeda, K., Nakanishi, M., *et al.* (1999). Cell cycle-dependent and ATM-independent expression of human Chk1 kinase. Oncogene *18*, 3673-3681.

Kapeluich, Y.L., Rubtsova, M., and Egorov, A.M. (1997). Enhanced chemiluminescence reaction applied to the study of horseradish peroxidase stability in the course of p-iodophenol oxidation. Journal of bioluminescence and chemiluminescence *12*, 299-308.

Karp, J.E., Thomas, B.M., Greer, J.M., Sorge, C., Gore, S.D., Pratz, K.W., Smith, B.D., Flatten, K.S., Peterson, K., Schneider, P., *et al.* (2012). Phase I and pharmacologic trial of cytosine arabinoside with the selective checkpoint 1 inhibitor Sch 900776 in refractory acute leukemias. Clin Cancer Res *18*, 6723-6731.

Kaufman, D.S., Shipley, W.U., and Feldman, A.S. (2009). Bladder cancer. The Lancet *374*, 239-249.

Kim, B.B., Pisarev, V.V., and Egorov, A.M. (1991). A comparative study of peroxidases from horse radish and Arthromyces ramosus as labels in luminol-mediated chemiluminescent assays. Analytical Biochemistry *199*, 1-6.

Kim, C., Snyder, R.O., and Wold, M.S. (1992). Binding properties of replication protein A from human and yeast cells. Mol Cell Biol *12*, 3050-3059.

Klionsky, D.J., Abdalla, F.C., Abeliovich, H., Abraham, R.T., Acevedo-Arozena, A., Adeli, K., Agholme, L., Agnello, M., Agostinis, P., Aguirre-Ghiso, J.A., *et al.* (2008). Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy *8*, 445-544.

Klionsky, D.J., Cuervo, A.M., and Seglen, P.O. (2007). Methods for Monitoring Autophagy from Yeast to Human. Autophagy *3*, 181-206.

Kohn, E.A., Ruth, N.D., Brown, M.K., Livingstone, M., and Eastman, A. (2002). Abrogation of the S Phase DNA Damage Checkpoint Results in S Phase Progression or Premature Mitosis Depending on the Concentration of 7-Hydroxystaurosporine and the Kinetics of Cdc25C Activation. Journal of Biological Chemistry *277*, 26553-26564.

Kohn, E.A., Yoo, C.J., and Eastman, A. (2003). The Protein Kinase C Inhibitor Go6976 Is a Potent Inhibitor of DNA Damage-induced S and G2 Cell Cycle Checkpoints. Cancer Research *63*, 31-35.

Kruhlak, M.J., Celeste, A., Dellaire, G., Fernandez-Capetillo, O., M 羹 ller, W.G., McNally, J.G., Bazett-Jones, D.P., and Nussenzweig, A. (2006). Changes in chromatin structure and mobility in living cells at sites of DNA double-strand breaks. The Journal of Cell Biology *172*, 823-834.

Kurz, E.U., and Lees-Miller, S.P. (2004). DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amst) *3*, 889-900.

Lavin, M.F. (2007). ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. Oncogene *26*, 7749-7758.

Lavin, M.F., and Kozlov, S. (2007). DNA damage-induced signalling in ataxia-telangiectasia and related syndromes. Radiotherapy and Oncology *83*, 231-237.

Lavin, M.F., and Shiloh, Y. (1997). The genetic defect in ataxia-telangiectasia. Annual review of immunology *15*, 177-202.

Lee, E.A., Keutmann, M.K., Dowling, M.L., Harris, E., Chan, G., and Kao, G.D. (2004). Inactivation of the mitotic checkpoint as a determinant of the efficacy of microtubule-targeted drugs in killing human cancer cells. Molecular Cancer Therapeutics *3*, 661-669.

Lee, J.H., and Paull, T.T. (2007). Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. Oncogene *26*, 7741-7748.

Letasiova, S., Medve'ova, A., Sovcikova, A., Dusinska, M., Volkovova, K., Mosoiu, C., and Bartonova, A. (2012). Bladder cancer, a review of the environmental risk factors. Environmental health : a global access science source *11 Suppl 1*, S11.

Li, W.Q., Pfeiffer, R.M., Hyland, P.L., Shi, J., Gu, F., Wang, Z., Bhattacharjee, S., Luo, J., Xiong, X., Yeager, M., *et al.* (2014). Genetic polymorphisms in the 9p21 region associated with risk of multiple cancers. Carcinogenesis.

Li, Y., Jenkins Cw Fau - Nichols, M.A., Nichols Ma Fau - Xiong, Y., and Xiong, Y. (1994). Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21.

Lin, C.W., Lin, J.C., and Prout, G.R., Jr. (1985). Establishment and characterization of four human bladder tumor cell lines and sublines with different degrees of malignancy. Cancer Res *45*, 5070-5079.

Lipinski, M.M., and Jacks, T. (1999). The retinoblastoma gene family in differentiation and development. Oncogene *18*, 7873-7882.

Liu, Q., Guntuku, S., Cui, X.-S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., *et al.* (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. Genes & Development *14*, 1448-1459.

Liu, S., Opiyo, S.O., Manthey, K., Glanzer, J.G., Ashley, A.K., Amerin, C., Troksa, K., Shrivastav, M., Nickoloff, J.A., and Oakley, G.G. (2012). Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. Nucleic Acids Research *40*, 10780-10794.

Los, M., Roodhart, J.M.L., and Voest, E.E. (2007). Target Practice: Lessons from Phase III Trials with Bevacizumab and Vatalanib in the Treatment of Advanced Colorectal Cancer. The Oncologist *12*, 443-450.

Lukas, C., Bartkova, J., Latella, L., Falck, J., Mailand, N., Schroeder, T., Sehested, M., Lukas, J., and Bartek, J. (2001). DNA Damage-activated Kinase Chk2 Is Independent of Proliferation or Differentiation Yet Correlates with Tissue Biology. Cancer Research *61*, 4990-4993.

Ma, C.X., Janetka, J.W., and Piwnica-Worms, H. (2011). Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. Trends in molecular medicine *17*, 88-96.

Mailand, N., Falck J Fau - Lukas, C., Lukas C Fau - Syljuasen, R.G., Syljuasen Rg Fau - Welcker, M., Welcker M Fau - Bartek, J., Bartek J Fau - Lukas, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage.

Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem *268*, 9194-9197.

Matsui, Y., Watanabe, J., Ding, S., Nishizawa, K., Kajita, Y., Ichioka, K., Saito, R., Kobayashi, T., Ogawa, O., and Nishiyama, H. (2010). Dicoumarol enhances doxorubicin-induced cytotoxicity in p53 wild-type urothelial cancer cells through p38 activation. BJU International *105*, 558-564.

Matsuoka, S., Huang, M., and Elledge, S.J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

Maya-Mendoza, A., Petermann, E., Gillespie, D.A.F., Caldecott, K.W., and Jackson, D.A. (2007). Chk1 regulates the density of active replication origins during the vertebrate S phase. EMBO J *26*, 2719-2731.

McGowan, C.H. (2002). Checking in on Cds1 (Chk2): A checkpoint kinase and tumor suppressor. BioEssays *24*, 502-511.

McNeely, S., Conti, C., Sheikh, T., Patel, H., Zabludoff, S., Pommier, Y., Schwartz, G., and Tse, A. (2010). Chk1 inhibition after replicative stress activates a double strand break response mediated by ATM and DNA-dependent protein kinase. Cell Cycle *9*, 995-1004.

Melo, J., and Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. Current Opinion in Cell Biology *14*, 237-245.

Meuth, M. (2010). Chk1 suppressed cell death. Cell Division *5*, 21.

Mirzayans, R., Andrais, B., Scott, A., and Murray, D. (2012). New insights into p53 signaling and cancer cell response to DNA damage: implications for cancer therapy. Journal of biomedicine & biotechnology *2012*, 170325.

Mizuno, K., Noda, K., Ueda, Y., Hanaki, H., Saido, T.C., Ikuta, T., Kuroki, T., Tamaoki, T., Hirai, S., Osada, S., *et al.* (1995). UCN-01, an anti-tumor drug, is a selective inhibitor of the conventional PKC subfamily. FEBS letters *359*, 259-261.

Myers, K., Gagou, M.E., Zuazua-Villar, P., Rodriguez, R., and Meuth, M. (2009). ATR and Chk1 Suppress a Caspase-3? ependent Apoptotic Response Following DNA Replication Stress. PLoS Genet *5*, e1000324.

Nakano, K., and Vousden, K.H. (2001). PUMA, a Novel Proapoptotic Gene, Is Induced by p53. Molecular Cell *7*, 683-694.

Negrini, S., Gorgoulis, V.G., and Halazonetis, T.D. (2010). Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol *11*, 220-228.

Ni, Z.-J., Barsanti, P., Brammeier, N., Diebes, A., Poon, D.J., Ng, S., Pecchi, S., Pfister, K., Renhowe, P.A., Ramurthy, S., *et al.* (2006). 4-(Aminoalkylamino)-3-benzimidazole-quinolinones as potent CHK-1 inhibitors. Bioorganic & Medicinal Chemistry Letters *16*, 3121-3124.

Niida, H., and Nakanishi, M. (2006). DNA damage checkpoints in mammals. Mutagenesis *21*, 3-9.

Nuss, J.E., Patrick, S.M., Oakley, G.G., Alter, G.M., Robison, J.G., Dixon, K., and Turchi, J.J. (2005). DNA damage induced hyperphosphorylation of replication protein A. 1. Identification of novel sites of phosphorylation in response to DNA damage. Biochemistry *44*, 8428-8437.

O'Toole, C.M., Povey, S., Hepburn, P., and Franks, L.M. (1983). Identity of some human bladder cancer cell lines. Nature *301*, 429-430.

Painter, R.B., and Young, B.R. (1980). Radiosensitivity in ataxia-telangiectasia: a new explanation. Proc Natl Acad Sci U S A *77*, 7315-7317.

Pan, Z.Q., Amin, A.A., Gibbs, E., Niu, H.W., and Hurwitz, J. (1994). PHOSPHORYLATION OF THE P34 SUBUNIT OF HUMAN SINGLE-STRANDED-DNA-BINDING PROTEIN IN CYCLIN-A-ACTIVATED G(1) EXTRACTS IS CATALYZED BY CDK CYCLIN-A COMPLEX AND DNA-DEPENDENT PROTEIN-KINASE. Proceedings of the National Academy of Sciences of the United States of America *91*, 8343-8347.

Pasion, S.G., Brown, G.W., Brown, L.M., and Ray, D.S. (1994). PERIODIC EXPRESSION OF NUCLEAR AND MITOCHONDRIAL-DNA REPLICATION GENES DURING THE TRYPANOSOMATID CELL-CYCLE. J Cell Sci *107*, 3515-3520.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-1505.

Petermann, E., and Helleday, T. (2010). Pathways of mammalian replication fork restart. Nat Rev Mol Cell Biol *11*, 683-687.

Petermann, E., Maya-Mendoza, A., Zachos, G., Gillespie, D.A.F., Jackson, D.A., and Caldecott, K.W. (2006). Chk1 Requirement for High Global Rates of Replication Fork Progression during Normal Vertebrate S Phase. Mol Cell Biol *26*, 3319-3326.

Petersen, L., Hasvold, G., Lukas, J., Bartek, J., and Syljuasen, R.G. (2010). p53-dependent G(1) arrest in 1st or 2nd cell cycle may protect human cancer cells from cell death after treatment with ionizing radiation and Chk1 inhibitors. Cell proliferation *43*, 365-371.

Platt, O.S. (2008). Hydroxyurea for the treatment of sickle cell anemia. The New England journal of medicine *358*, 1362-1369.

Pompella, A., Visvikis, A., Paolicchi, A., Tata, V.D., and Casini, A.F. (2003). The changing faces of glutathione, a cellular protagonist. Biochemical Pharmacology *66*, 1499-1503.

Qamar, L., Davis, R., Anwar, A., and Behbakht, K. (2008). Protein kinase C inhibitor Go6976 augments caffeine-induced reversal of chemoresistance to cis-diamminedichloroplatinum-II (CDDP) in a human ovarian cancer model. Gynecologic oncology *110*, 425-431.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Risau, W., and Flamme, I. (1995). Vasculogenesis. Annual review of cell and developmental biology *11*, 73-91.

Roberts, P.J., and Der, C.J. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene *26*, 3291-3310.

Rodriguez, R., Gagou, M.E., and Meuth, M. (2008). Apoptosis induced by replication inhibitors in Chk1-depleted cells is dependent upon the helicase cofactor Cdc45. Cell Death Differ *15*, 889-898.

Rodriguez, R., and Meuth, M. (2006). Chk1 and p21 Cooperate to Prevent Apoptosis during DNA Replication Fork Stress. Molecular Biology of the Cell *17*, 402-412.

Rogakou, E.P., Boon, C., Redon, C., and Bonner, W.M. (1999). Megabase Chromatin Domains Involved in DNA Double-Strand Breaks in Vivo. The Journal of Cell Biology *146*, 905-916.

Saad, A., Hanbury, D., McNicholas, T., Boustead, G., Morgan, S., and Woodman, A. (2002). A study comparing various noninvasive methods of detecting bladder cancer in urine. BJU International *89*, 369-373.

Sacerdote, C., Guarrera, S., Ricceri, F., Pardini, B., Polidoro, S., Allione, A., Critelli, R., Russo, A., Andrew, A.S., Ye, Y., *et al.* (2013). Polymorphisms in the XRCC1 gene modify survival of bladder cancer patients treated with chemotherapy. International journal of cancer Journal international du cancer *133*, 2004-2009.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S.J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science *277*, 1497-1501.

Sausville, E.A., Arbuck, S.G., Messmann, R., Headlee, D., Bauer, K.S., Lush, R.M., Murgo, A., Figg, W.D., Lahusen, T., Jaken, S., *et al.* (2001). Phase I Trial of 72-Hour Continuous Infusion UCN-01 in Patients With Refractory Neoplasms. Journal of Clinical Oncology *19*, 2319-2333.

Savitsky, K., Sfez, S., Tagle, D.A., Ziv, Y., Sartiel, A., Collins, F.S., Shiloh, Y., and Rotman, G. (1995). The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. Hum Mol Genet *4*, 2025-2032.

Sentein, P. (1977). Action of nocodazole on the mechanisms of segmentation mitosis. . Cell Biol Int Rep *1*, 503-509.

Seto, T., Esaki, T., Hirai, F., Arita, S., Nosaki, K., Makiyama, A., Kometani, T., Fujimoto, C., Hamatake, M., Takeoka, H., *et al.* (2013). Phase I, dose-escalation study of AZD7762 alone and in combination with gemcitabine in Japanese patients with advanced solid tumours. Cancer Chemother Pharmacol *72*, 619-627.

Shao, R.-G., Cao, C.-X., and Pommier, Y. (1997). Activation of PKCa Downstream from Caspases during Apoptosis Induced by 7-Hydroxystaurosporine or the Topoisomerase Inhibitors, Camptothecin and Etoposide, in Human Myeloid Leukemia HL60 Cells. Journal of Biological Chemistry *272*, 31321-31325.

Shariat, S.F., Sfakianos, J.P., Droller, M.J., Karakiewicz, P.I., Meryn, S., and Bochner, B.H. (2010). The effect of age and gender on bladder cancer: a critical review of the literature. BJU International *105*, 300-308.

Shechter, D., Costanzo, V., and Gautier, J. (2004). ATR and ATM regulate the timing of DNA replication origin firing. Nature cell biology *6*, 648-655.

Shi, Z., Azuma, A., Sampath, D., Li, Y.-X., Huang, P., and Plunkett, W. (2001). S-Phase Arrest by Nucleoside Analogues and Abrogation of Survival without Cell Cycle Progression by 7-Hydroxystaurosporine. Cancer Research *61*, 1065-1072.

Shimada, M., Niida, H., Zineldeen, D.H., Tagami, H., Tanaka, M., Saito, H., and Nakanishi, M. (2008). Chk1 Is a Histone H3 Threonine 11 Kinase that Regulates DNA Damage-Induced Transcriptional Repression. Cell *132*, 221-232.

Sidi, S., Sanda, T., Kennedy, R.D., Hagen, A.T., Jette, C.A., Hoffmans, R., Pascual, J., Imamura, S., Kishi, S., Amatruda, J.F., *et al.* (2008). Chk1 Suppresses a Caspase-2 Apoptotic Response to DNA Damage that Bypasses p53, Bcl-2, and Caspase-3. Cell *133*, 864-877.

Siegel, R., Ma, J., Zou, Z., and Jemal, A. (2014). Cancer statistics, 2014. CA Cancer J Clin *64*, 9-29.

Slater, M.L. (1973). Effect of Reversible Inhibition of Deoxyribonucleic Acid Synthesis on the Yeast Cell Cycle. Journal of Bacteriology *113*, 263-270.

Smits, V.A., Reaper, P.M., and Jackson, S.P. (2006). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Current biology : CB *16*, 150-159.

Soloway, M.S., Sofer, M., and Vaidya, A. (2002). Contemporary Management Of Stage T1 Transitional Cell Carcinoma Of The Bladder. The Journal of Urology *167*, 1573-1583.

Sorensen, C.S., Hansen, L.T., Dziegielewski, J., Syljuasen, R.G., Lundin, C., Bartek, J., and Helleday, T. (2005). The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. Nature cell biology *7*, 195-201.

Stiff, T., Walker, S.A., Cerosaletti, K., Goodarzi, A.A., Petermann, E., Concannon, P., O'Driscoll, M., and Jeggo, P.A. (2006). ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. EMBO J *25*, 5775-5782.

Stracker, T.H., Usui, T., and Petrini, J.H.J. (2009). Taking the time to make important decisions: The checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. DNA Repair *8*, 1047-1054.

Stravopodis, D.J., Karkoulis, P.K., Konstantakou, E.G., Melachroinou, S., Thanasopoulou, A., Aravantinos, G., Margaritis, L.H., Anastasiadou, E., and Voutsinas, G.E. (2011). Thymidylate synthase inhibition induces p53-dependent and p53-independent apoptotic responses in human urinary bladder cancer cells. Journal of cancer research and clinical oncology *137*, 359-374.

Syljuasen, R.G., Sorensen, C.S., Hansen, L.T., Fugger, K., Lundin, C., Johansson, F., Helleday, T., Sehested, M., Lukas, J., and Bartek, J. (2005). Inhibition of Human Chk1 Causes Increased Initiation of DNA Replication, Phosphorylation of ATR Targets, and DNA Breakage. Mol Cell Biol *25*, 3553-3562.

Sylvester, R.J., van der Meijden, A.P.M., and Lamm, D.L. (2002). Intravesical Bacillus Calmette-Guerin Reduces the Risk of Progression in Patients with Superficial Bladder Cancer: A Meta-analysis of the Published Results of Randomized Clinical Trials. The Journal of Urology *168*, 1964-1970.

Takai, H., Naka, K., Okada, Y., Watanabe, M., Harada, N., Saito, S., Anderson, C.W., Appella, E., Nakanishi, M., Suzuki, H., *et al.* (2002). Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. EMBO J *21*, 5195-5205.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y.A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakanishi, M., and Nakayama, K.-i. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1 ????mice. Genes & Development *14*, 1439-1447.

Tavera-Mendoza, L., Wang, T.T., Lallemant, B., Zhang, R., Nagai, Y., Bourdeau, V., Ramirez-Calderon, M., Desbarats, J., Mader, S., and White, J.H. (2006). Convergence of vitamin D and retinoic acid signalling at a common hormone response element. EMBO reports *7*, 180-185.

Tomkiel, J.E., Alansari, H., Tang, N., Virgin, J.B., Yang, X., VandeVord, P., Karvonen, R.L., Granda, J.L., Kraut, M.J., Ensley, J.F., *et al.* (2002). Autoimmunity to the Mr 32,000 Subunit of Replication Protein A in Breast Cancer. Clinical Cancer Research *8*, 752-758.

Toschi, L., Finocchiaro, G., Bartolini, S., Gioia, V., and Cappuzzo, F. (2005). Role of gemcitabine in cancer therapy. Future Oncology *1*, 7-17.

Tse, A.N., Carvajal, R., and Schwartz, G.K. (2007a). Targeting Checkpoint Kinase 1 in Cancer Therapeutics. Clinical Cancer Research *13*, 1955-1960.

Tse, A.N., Rendahl, K.G., Sheikh, T., Cheema, H., Aardalen, K., Embry, M., Ma, S., Moler, E.J., Ni, Z.J., Lopes de Menezes, D.E., *et al.* (2007b). CHIR-124, a Novel Potent Inhibitor of Chk1, Potentiates the Cytotoxicity of Topoisomerase I Poisons In vitro and In vivo. Clinical Cancer Research *13*, 591-602.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M., and Wahl, G.M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell *9*, 1031-1044.

van Rhijn, B.W., Catto, J.W., Goebell, P.J., Knuchel, R., Shariat, S.F., van der Poel, H.G., Sanchez-Carbayo, M., Thalmann, G.N., Schmitz-Drager, B.J., and Kiemeney, L.A. (2014). Molecular markers for urothelial bladder cancer prognosis: Toward implementation in clinical practice. Urologic oncology.

Vassin, V.M., Anantha, R.W., Sokolova, E., Kanner, S., and Borowiec, J.A. (2009). Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. J Cell Sci *122*, 4070-4080.

Vassin, V.M., Wold, M.S., and Borowiec, J.A. (2004). Replication Protein A (RPA) Phosphorylation Prevents RPA Association with Replication Centers. Mol Cell Biol *24*, 1930-1943.

Verlinden, L., Vanden Bempt, I., Eelen, G., Drijkoningen, M., Verlinden, I., Marchal, K., De Wolf-Peeters, C., Christiaens, M.-R., Michiels, L., Bouillon, R., *et al.* (2007). The E2F-Regulated Gene Chk1 Is Highly Expressed in Triple-Negative Estrogen Receptor/Progesterone Receptor/HER-2 Breast Carcinomas. Cancer Research *67*, 6574-6581.

Walker, M., Black, E.J., Oehler, V., Gillespie, D.A., and Scott, M.T. (2009). Chk1 C-terminal regulatory phosphorylation mediates checkpoint activation by de-repression of Chk1 catalytic activity. Oncogene *28*, 2314-2323.

Wang, B., Li, Z., Wang, C., Chen, M., Xiao, J., Wu, X., Xiao, W., Song, Y., and Wang, X. (2013). Zygotic G2/M cell cycle arrest induced by ATM/Chk1 activation and DNA repair in mouse embryos fertilized with hydrogen peroxide-treated epididymal mouse sperm. PLoS One *8*, e73987.

Wang, Q., Fan, S., Eastman, A., Worland, P.J., Sausville, E.A., and O'Connor, P.M. (1996). UCN-01: a Potent Abrogator of G2 Checkpoint Function in Cancer Cells With Disrupted p53. Journal of the National Cancer Institute *88*, 956-965.

Wang, Y., Putnam, C.D., Kane, M.F., Zhang, W., Edelmann, L., Russell, R., Carrion, D.V., Chin, L., Kucherlapati, R., Kolodner, R.D., *et al.* (2005). Mutation in Rpa1 results in defective DNA double-strand break repair, chromosomal instability and cancer in mice. Nature genetics *37*, 750-755.

Watanabe, J., Nishiyama, H., Okubo, K., Takahashi, T., Toda, Y., Habuchi, T., Kakehi, Y., Tada, M., and Ogawa, O. (2004). Clinical evaluation of p53 mutations in urothelial carcinoma by IHC and FASAY. Urology *63*, 989-993.

Wilsker, D., Petermann, E., Helleday, T., and Bunz, F. (2008). Essential function of Chk1 can be uncoupled from DNA damage checkpoint and replication control. Proceedings of the National Academy of Sciences *105*, 20752-20757.

Wobbe, C.R., Weissbach, L., Borowiec, J.A., Dean, F.B., Murakami, Y., Bullock, P., and Hurwitz, J. (1987). Replication of simian virus 40 origin-containing DNA in vitro with purified proteins. Proceedings of the National Academy of Sciences *84*, 1834-1838.

Wold, M.S. (1997). Replication protein A: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Biochem *66*, 61-92.

Wold, M.S., and Kelly, T. (1988). Purification and characterization of replication protein A, a cellular protein required for in vitro replication of simian virus 40 DNA. Proceedings of the National Academy of Sciences *85*, 2523-2527.

Wold, M.S., Weinberg, D.H., Virshup, D.M., Li, J.J., and Kelly, T.J. (1989). IDENTIFICATION OF CELLULAR PROTEINS REQUIRED FOR SIMIAN VIRUS-40 DNA-REPLICATION. Journal of Biological Chemistry *264*, 2801-2809.

Wood, M.D., and Sanchez, Y. (2010). Deregulated Ras signaling compromises DNA damage checkpoint recovery in S. cerevisiae Cell Cycle *9*, 3353 - 3363.

Wu, W., Fan, Y.-H., Kemp, B.L., Walsh, G., and Mao, L. (1998). Overexpression of cdc25A and cdc25B Is Frequent in Primary Non-Small Cell Lung Cancer but Is Not Associated with Overexpression of c-myc. Cancer Research *58*, 4082-4085.

Xiao, Z., Chen, Z., Gunasekera, A.H., Sowin, T.J., Rosenberg, S.H., Fesik, S., and Zhang, H. (2003). Chk1 Mediates S and G2 Arrests through Cdc25A Degradation in Response to DNA-damaging Agents. Journal of Biological Chemistry *278*, 21767-21773.

Xie, H., Gong, Y., Dai, J., Wu, X., and Gu, J. (2013). Genetic variations in base excision repair pathway and risk of bladder cancer: A case-control study in the United States. Molecular carcinogenesis.

Yoo, H.Y., Kumagai, A., Shevchenko, A., Shevchenko, A., and Dunphy, W.G. (2007). Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM. J Biol Chem *282*, 17501-17506.

You, Z.Z., Kong, L., and Newport, J. (2002). The role of single-stranded DNA and polymerase alpha in establishing the ATR, Hus1 DNA replication checkpoint. Journal of Biological Chemistry *277*, 27088-27093.

Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W., and Vogelstein, B. (2001). PUMA Induces the Rapid Apoptosis of Colorectal Cancer Cells. Molecular Cell *7*, 673-682.

Zachos, G., Rainey, M.D., and Gillespie, D.A.F. (2003). Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. EMBO J *22*, 713-723.

ZernikKobak, M., Vasunia, K., Connelly, M., Anderson, C.W., and Dixon, K. (1997). Sites of UV-induced phosphorylation of the p34 subunit of replication protein A from HeLa cells. Journal of Biological Chemistry *272*, 23896-23904.

Zhang, Y., Jones, T., Martin, S., Caplen, N., and Pommier, Y. (2009). Implication of checkpoint kinase-dependent up-regulation of ribonucleotide reductase R2 in DNA damage response. J Biol Chem *284*, 18085 - 18095.

Zhao, H., and Piwnica-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Molecular and cellular biology *21*, 4129-4139.

Zhao, H., Watkins, J.L., and Piwnica-Worms, H. (2002). Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci U S A *99*, 14795-14800.

Zou, L., and Elledge, S.J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.

Zou, Y., Liu, Y., Wu, X., and Shell, S.M. (2006). Functions of human replication protein A (RPA): From DNA replication to DNA damage and stress responses. Journal of Cellular Physiology *208*, 267-273.