I, Emily Davis, hereby declare that the work on which this thesis is based is my original work (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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August, 2009

Acknowledgements

This thesis is dedicated to the following people who have helped shape my future:

My dad and Jill. Your continued love, guidance and support are deeply valued.

My mother who, in her limited time, taught me so much.

My supervisor, mentor and friend, Sharon Prince. I thank you for all the knowledge, time and support you have given me and your unwavering enthusiasm for science. You are a true inspiration.

Sally, Nicki and Duncan, for their encouragement and for being such great people. It has been a privilege to have family as friends.

I am thankful to all my fellow lab members, past and present, for creating an enjoyable working environment, for critical feedback on my work and help with experiments. I especially thank Amaal, Shaheen and Deeya (the original T-box girls) not only for their insightful discussions, but also for their wonderful friendship.

I am particularly grateful to all my friends, especially Nadya and Deborah, for emotional support, comic relief and being so understanding of the countless times that I was late due to time delays in the lab.

I wish to thank:
Dr Colin Goding for allowing me to spend three months in his laboratory at the Marie Curie Research Institute, UK and for providing reagents that contributed to this study.

Professor Dhiren Govender and Dr Bleda Bilican for academic support.

The National Research Foundation, German Academic Exchange Service and the University of Cape Town who provided financial assistance during the period of study towards this degree.

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Abstract

The role of the developmentally important transcription factor, TBX2, in the cell cycle and cancer

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August 2009

T-box factors play crucial roles in embryogenesis and mutations in T-box factor genes have been implicated in multiple human disorders. In addition, an increasing body of evidence implicates the T-box family in cell cycle regulation and in cancer. For example, TBX2 can suppress senescence through repressing $p19^{ARF}$ and $p21^{WAF1/CIP1/SDI1}$ gene expression and is over-expressed in melanoma, breast and pancreatic cancers. Although these studies suggest that TBX2 may contribute to the oncogenic process, whether TBX2 plays a causative role in this process and whether this exclusively involves its anti-senescence function has not yet been elucidated. The aim of this study was therefore to address this question by establishing TBX2 over-expression and knockdown cell culture models. The results show that TBX2 does indeed contribute directly to the oncogenic process and further reveals a novel mechanism by which it contributes to tumourigenesis.

Over-expression of TBX2 in transformed human lung fibroblast cells resulted in severe morphological alterations. Indeed, flow cytometry and metaphase spread assays showed that a sub-population of TBX2-expressing cells failed to undergo cytokinesis and proceeded through the cell cycle undetected, containing double the number of chromosomes. Immunofluorescence analyses revealed that the TBX2-expressing cells were larger, with binucleate and lobular nuclei. Furthermore, G-banding and immunofluorescence analyses were utilised to show that the TBX2-expressing cells had increased frequency of several features of genomic instability such as chromosome missegregation and chromosomal rearrangements. While grossly abnormal, these cells were still capable of dividing and gave rise to cells that were resistant to the chemotherapeutic drug cisplatin.

Knocking down TBX2 was shown to be sufficient to reduce several features of transformation in both a breast cancer and melanoma cell line which express high levels of this protein. In particular, growth curves and colony formation assays revealed that knocking down TBX2 reduced the proliferation rate and re-established the requirement for mitogenic stimuli and a substrate. The results suggest that TBX2 is a powerful pro-proliferative factor, particularly under growth-inhibitory conditions. Furthermore, knocking down TBX2 sensitised the MCF-7 breast cancer cells to cisplatin by abrogating a p53-dependent S-phase arrest and driving cells into a mitotic catastrophe. The effect of cisplatin treatment on cell survival when TBX2 was either knocked down or over-expressed suggests that TBX2 may enhance chemotherapeutic drug resistance in TBX2-associated cancers. Taken together results from this study demonstrate that TBX2 is directly involved in the oncogenic process and that it is an attractive novel target in the development of anti-cancer drugs.

Chapter 1 Literature Review

1.1 *Introduction*

Cancer arises from a single cell through progressive acquisition of genetic mutations that ultimately derails the cell cycle leading to uncontrolled cell proliferation. In the last 50 years the advent of techniques to study molecular biology has led to huge progress in an understanding of the molecular origins and progression of diseases such as cancer. Unlike the positive impact of these advances on major diseases such as coronary heart disease and stroke, the incidence of cancer continues to increase globally and it is one of the leading causes of death worldwide. One of the major obstacles facing cancer research, and in particular the development of effective treatments, is the multifactorial nature of this disease as well as the diverse characteristics among tumours of even the same origin. A greater understanding of the molecular mechanisms regulating the cell cycle and its deregulation during tumourigenesis is therefore critical to the elucidation of the events underpinning cancer development. This has important implications for the identification of effective cancer treatments.

Several processes which occur during development such as cell proliferation and migration are also characteristics of cancer cells. It is therefore not surprising that many genes involved in development have been found to be deregulated during tumourigenesis. A good example of this is the T-box family of transcription factors which were originally identified for their central role in development and have more recently been implicated in tumourigenesis. While the critical role of T-box factors in development, which ranges from cell fate determination to organogenesis, has been well described very little information is still available on their role in tumourigenesis. Much of the evidence linking T-box factors to cancer stem from studies on two members of this family, the closely related TBX2[1] and TBX3. For example, TBX2 and TBX3 can suppress senescence (see section 1.3.3) and are over-expressed in several cancers including melanoma, breast and pancreatic cancers (Jacobs et al., 2000; Carlson et al., 2001;

[1] The accepted convention will be used for human, TBX2, and when referring to both human and mouse, TBX2/Tbx2 will be used.

Brummelkamp et al., 2002a; Prince et al., 2004, Packham and Brook, 2003; Sinclair et al., 2002 ; Fan et al., 2004; Hoek et al., 2004; Yarosh et al., 2008; Hansel et al., 2004; Lomnytska et al., 2005; Chen et al., 2009). This review will provide a general overview of the key areas of research pertaining to this thesis.

1.2 *The T-box gene family*

The *Brachyury* or *T* gene is the prototype of the T-box gene family and was first described in mouse in 1927 (Dobrovolskaia-Zavadskaia, 1927) and orthologs have since been identified in a number of species including, zebrafish, Drosophila, chick, Xenopus and human (Kispert et al, 1995b; Knezevic et al., 1997; Smith et al., 1991; Schulte-Merker et al., 1992; Edwards et al., 1996). The discovery of *Brachyury* and its role in embryogenesis played a pioneering role, not only in the identification of the T-box family, but also in the field of developmental biology and genetics. This gene was initially identified in one of the first successful mammalian genetic screens, and illustrates one of the earliest links between gene function and cell behaviour during embryogenesis. In addition, the cloning of *Brachyury* 60 years later represents one of the first positional cloning endeavours in mouse. Brachyury is the most extensively studied T-box factor and has been utilised as a model for elucidating the structure and function of other family members. Results of some of these studies will therefore be described here.

Brachyury, also referred to as T, was identified due to a semidominant mutation in this gene resulting in a short tail phenotype in mice (Dobrovolskaia-Zavadskaia, 1927). '*T'* denotes 'tail' and this accounts for the name of the gene family. The integral role that *Brachyury* plays in embryogenesis is underscored by the observations that homozygous mutants die *in utero*, at about 10 days postcoitum. At this stage there is almost completely normal development in the anterior region of the mouse down to the buds of the forelimbs, however further development is arrested and the posterior region fails to develop into the trunk or tail (Chelsey, 1935). This malformation is due to improper development of the notochord, resulting in abnormal neural tube and somite development (Chelsey, 1935). A study by Glucksohn-Schonheimer in 1944 however established that the embryonic lethality was due to improper development of the allantois, a structure derived from the mesoderm which connects the embryo to the placenta of the mother.

Herrmann et al. cloned the *Brachyury* gene in 1990 and numerous studies carried out since have made an enormous contribution to our understanding of the structure and function of the *T* gene and its gene product. The T protein functions as a transcription factor and its N-terminal domain was shown to bind the palindromic sequence T(G/C)ACACCTAGGTGTGAAATT (subsequently referred to as the T site) as a monomer, but preferentially as a dimer (Kispert and Herrmann, 1993; Kispert et al., 1995a). Furthermore, studies using Brachyury deletion constructs identified the N-terminal residues 18–229 to be critical for DNA binding (Kispert and Herrmann, 1993). The T protein has two transactivation domains and it has been shown to function as a transcriptional activator. Further studies, however, revealed the presence of two repression domains in the C-terminus (Kispert et al, 1995a), suggesting that the protein may have the ability to function as both an activator and a repressor.

Brachyury has been shown to display dosage sensitivity. Different mutant alleles of this gene result in mice displaying varying short tail phenotypes, which led to the suggestion that increased axial formation requires increasingly higher doses of the T protein (MacMurray and Shin, 1988). This was confirmed in experiments in which the wild type T-protein was introduced into the germ line of a wild type *T*- mouse, which when bred against various mutant backgrounds was able to rescue the mutant phenotype in a dose–dependent manner (Stott et al., 1993). This work showed specifically that increased copy number of the wild type *T*-gene correlates directly with greater axial development.

The possible existence of a T-box protein family first emerged with the identification of the *Drosophila omb* gene, and since then members have been identified in numerous species ranging from hydra to human (Pflugfelder et al., 1992; reviewed by Papaioannou, 2001). The family is divided into subfamilies, namely T, Tbx1, Tbx2, Tbx6 and T-brain1, based on similarities in expression patterns and evolutionary relatedness. All T-box proteins recognise the same palindromic *Brachyury* response element (T site) and contain at the very least a sequence-specific DNA-binding domain, the T-box, and a transcriptional activator and/or repressor domain with the relative position of these domains differing between different members of the family (Kispert and Herrmann, 1993; reviewed by Wilson and Conlon, 2002). Despite the observation that all T-box factors tested to date recognise the same T site,

they play distinct roles which suggest that they regulate different target genes (Sinha et al., 2000). It is now generally agreed that the target gene specificity for T-box factors rely on their distinct association with cofactors and that this may play an important role in determining whether T-box proteins function as activators or repressors of their target genes (Tada and Smith, 2001). For example, T-brain-1 forms a complex with two proteins in the nucleus; Calcium/calmodulin-dependent serine protein kinase (*CASK)* and CASK- Interacting Nucleosome Assembly Protein, resulting in an enhanced ability of T-brain-1 to transcriptionally activate its target genes such as NMDAR subunit 2b (Hsueh et al. 2000; Wang et al., 2004b).

Consistent with their important role in development, T-box family members are highly conserved during evolution. They are required for the development of several cell types and play critical roles in processes ranging from gastrulation to organogenesis (reviewed by Wilson and Conlon, 2002; Showell et al., 2004). The importance of their role in development is further emphasised by observations that mutations in many human T-box genes result in developmental defects. For example, mutations in *TBX1* are associated with DiGeorge syndrome, which is characterised by cardiovascular, thymus and parathyroid defects as well as some craniofacial anomalies (Jerome and Papaioannou, 2001; reviewed by Packham and Brook, 2003). Haploinsufficiency of *TBX3* and *TBX5* result in Ulnar-Mammary and Holt-Oram syndromes respectively (Bamshad et al., 1997; Basson et al., 1997). Ulnar-mammary syndrome (UMS) affects limb development with abnormalities ranging from hypoplasia of the fifth digit to some individuals completely lacking the hand and forearm. In addition, individuals suffering from UMS have abnormalities in the development of the teeth, breast and genitalia (reviewed by Packham and Brook, 2003). The degree of the deformities observed in patients with UMS varies depending on the effect of the mutation on the functional activity of TBX3. Individuals with Holt-Oram syndrome display varying phenotypes involving skeletal malformation such as hand, wrist and/or arm deformities as well as cardiac abnormalities. The expression of *TBX19,* also known as *Tpit,* is necessary for the expression of pro-opiomelanocortin (POMC) in the pituitary and mutations in this gene results in adrenal insufficiency (Liu et al., 2001). Furthermore, X-linked cleft palate and ankyloglossia can result from mutations in *TBX22* (Braybrook et al., 2001). Evidence also points towards *TBX15* being a candidate for the dominantly expressed acromegaloid facial appearance syndrome (Agulnik et al, 1998). Due to its relevance to this study the following

section of this review will provide a general overview of the current literature available on TBX2 with a special focus on its role in the cell cycle and cancer.

1.3 *TBX2*

1.3.1 Gene and protein

TBX2 was the first T-box factor identified in human as well as the first member shown to function as a transcriptional repressor (Campbell et al., 1995; Carreira et al., 1998). The TBX2 subfamily consists of 4 T-box factors, namely *TBX2, TBX3, TBX4* and *TBX5*, which originated from one common ancestral gene (Agulnik et al., 1996). Duplication and recombination of a two-gene cluster, which itself originally stemmed from unequal crossing over of one ancestral gene, resulted in *TBX2* and *TBX4* being positioned on chromosome 17q23 and *TBX3* and *TBX5* being positioned on chromosome 12q24 (Agulnik et al., 1996). Due to this crossing over event, *TBX2* is more closely related to *TBX3* and *TBX4* is more closely related to *TBX5*.

The human *TBX2* coding region, which contains 7 exons, spans 3.378 kb and encodes a protein of 712 amino acids. The DNA binding domain is located within the N-terminal of the protein, at amino acids 106-289. Site directed mutagenesis demonstrated arginine 122 to be essential for DNA binding and indeed homology modelling studies on the Brachyury crystallographic structure confirmed reports that this arginine residue is conserved in all T-box proteins identified to date (Sinha et al., 2000).

The Tbx2 protein has been shown to bind the palindromic consensus T sequence in the promoters of target genes as a monomer rather than as a dimer (Sinha et al., 2000) and target gene binding is required for their repression (Carreira et al., 1998; Paxton et al., 2002). Tbx2 can repress its targets via both an N- and C-terminal repression domain, located between amino acids 1 and 53 and 529 and 573 respectively, and a weak activation domain was also identified within the T-box (Paxton et al., 2002). While a number of genes have been identified that are repressed by Tbx2, to date no genes have been shown to be directly activated by Tbx2 *in vivo*.

1.3.2 TBX2 target genes in development

Tbx2 functions to control cell differentiation, proliferation and fate through transcriptionally repressing its target genes. The role of Tbx2 in development is not the focus of the current study and therefore this will be described briefly, using specific examples where direct Tbx2 targets have been identified.

During development, Tbx2 is expressed in a diverse group of organs and tissues including the limb, kidney, lung, mammary gland, heart, prostate, spleen, testis, thymus, placenta and ovaries (Bollag et al., 1994; Campbell, et el., 1995; Law et al., 1995). TBX2 may also be important in postnatal gene regulation because it is expressed in the adult kidney, lung, prostate, spleen and ovaries (Law et al., 1995). While TBX2 has been linked to the development of several tissues and organs, its expression has been shown to be required for proper limb development and digit identity and is necessary for proliferation and patterning of the developing heart (Harrelson et al., 2004; Suzuki et al., 2004; Nissim et al., 2007). To date no developmental syndrome has been associated with *TBX2* mutations and mice with heterozygous null mutations appear normal (Harrelson et al., 2004). However, observations that homozygous mutations are lethal suggest a crucial role for TBX2 in development (Harrelson et al., 2004).

Tbx2 expression has been observed in several melanocyte and melanoma cell lines but not in pre-melanoblast cells (Carreira et al., 1998). Melanoblasts are neural crest derived cells which migrate to the hair follicles and epidermis where they differentiate into mature melanocytes capable of producing pigment (Silvers, 1979). The murine Tbx2 was cloned by Carreira et al. (1998) and shown to repress the melanocyte-specific *tyrosinase-related protein 1* (*TYRP-1*). TYRP-1 has a well described enzymatic function in the melanin biosynthesis pathway, and more recent data would suggest that it also plays a role as a positive regulator of melanocyte proliferation (Sarangarajan et al., 2000). Since Tbx2 is not involved in melanin production and, if anything, promotes proliferation by bypassing senescence, the functional significance of *TYRP-1* repression by Tbx2 remains unclear.

Recent studies have identified several Tbx2 target genes that play a role during early heart development. The vertebrate heart originates from a linear tube that undergoes a series of complex looping and partitioning to form the multi-chambered organ. At the onset of

looping, the heart tube is compartmentalised into chamber myocardium and non-chamber myocardium and together these form the cardiac conduction system, which essentially controls the heart rate (Christoffels et al., 2004a). The formation of the chamber and non-chamber myocardium requires a distinct set of genes, and a number of chamber and non-chamber specific genes have been characterised. For example, expression of *natriuretic precursor peptide type A* (*Nppa*), *connexin* (*Cx*) *40*, and *Cx43* are important for the differentiation and formation of the chamber myocardium (Christoffels et al., 2000; Christoffels et al., 2004b). Formation of the non-chamber myocardium on the other hand requires the expression of a different set of genes which involves Tbx2 (Christoffels et al., 2004b; Habets et al., 2002). Tbx2 normally inhibits cell proliferation and chamber differentiation in non-chamber myocardium, and it is thought that chamber differentiation requires repression of *Tbx2* by Tbx20 (Cai et al., 2005). Tbx20, a positive regulator of chamber formation, binds and transcriptionally represses *Tbx2* via a T-element (Cai et al., 2005). Given the ability of Tbx2 to repress *Nmyc1*, which is required for the normal proliferation of the heart (Davis and Bradley, 1993), it is proposed that in chamber myocardium Tbx20 represses *Tbx2* thus preventing its repression of *Nmyc1* (Cai et al., 2005). However, in non-chamber myocardium repression of *Tbx2* by Tbx20 is abolished, allowing Tbx2 to repress *Nmyc1* and the early cardiac genes, resulting in decreased proliferation within this region (Cai et al., 2005; Stennard et al., 2005). *In vitro* reporter assays and transgenic mice studies have shown that during non-chamber myocardium formation, Tbx2 represses the transcription of *Nppa, Cx40,* and *Cx43* and thus plays a role in regulating the formation of the multi-chambered heart (Christoffels et al., 2004b Habets et al., 2002).

During heart development the non-chamber myocardium retains the embryonic myocardial phenotype of the tubular heart longer than the chamber myocardium. The *Nppa* gene is specifically expressed in the developing chamber myocardium and is one of the first hallmarks of chamber formation (Christoffels et al., 2000), which raises the question of how this gene is repressed in the non-chamber myocardium. This was addressed by studies which found that mutating two adjacent binding sites for T-box factors and Nkx2.5, respectively, abrogated repression of *Nppa* in regions of the non-chamber myocardium (Habets et al., 2002). Furthermore, Tbx2 was shown to co-operate with Nkx2.5 to suppress *Nppa* promoter activity in embryonic myocardium (Habets et al., 2002). Interestingly, the physical and functional interaction between Nkx2.5 and Tbx5 were shown to be involved in synergistically

activating an *Nppa*-reporter gene (Bruneau et al., 2001; Hiroi et al., 2001). Moreover, Tbx2 was shown to decrease Nkx2.5-Tbx5-mediated activation of the *Nppa* promoter (Habets et al., 2002). Taken together, researchers believe that *Nppa* expression is regulated by the competition between Tbx2 and Tbx5 to bind and cooperate with Nkx2.5.

Tbx2 has been shown to regulate expression of the genes encoding connexin (Cx) 43 and collagen, which are factors involved in bone formation. Gap junctions, composed of connexin protein subunits, function to connect the cytoplasm of adjacent bone (osteocytes) cells and are therefore important in cell-cell communication. Cx43 is the predominant gap junction protein in bone and its regulated temporal pattern of expression has been shown to play a critical role in normal ossification and osteoblast function (Lecanda et al., 2000). *Tbx2* and *Cx43* are co-expressed in osteogenic progenitors and osteoblasts, which suggested that Cx43 may be a potential Tbx2 target gene. This was tested in a study in which a rat osteosarcoma cell line was transfected with either sense or anti-sense Tbx2 and the results showed that inhibition of Tbx2 resulted in a marked increase in *Cx43* expression (Borke et al., 2003; Chen et al., 2004). The *Cx43* promoter has several T-element half sites and Tbx2 was shown to directly bind and repress the *Cx43* promoter at two of these sites (Chen et al., 2004). Interestingly, in the same study transgenic mice injected with the LacZ reporter cloned downstream of either the wild-type (WT) or mutant *Cx43* promoter in which the two T-elements were mutated exhibited the same expression pattern of LacZ. These results suggest that *in vivo*, Tbx2 may require co-factors to regulate *Cx43* expression. It is also important to note that these studies were done in mouse and rat, suggesting that the role of Tbx2 during bone development may vary between species.

Type 1 collagen synthesis is crucial for normal embryonic development and in maintaining tissue integrity, and its aberrant expression has deleterious effects on several biological processes including bone development (Bornstein and Sage, 1989). In an attempt to identify genes that may be regulated by Tbx2, cDNA microarray analysis was performed on mouse NIH 3T3 fibroblasts over-expressing *Tbx2* and results revealed that the *type 1 collagen* gene was upregulated (Chen et al., 2001). Interestingly, a parallel investigation in which *Tbx2* was over-expressed in the rat ROS17/2.8 osteoblastic cell line showed downregulation of *type 1 collagen* (Chen et al., 2001). A recent study in our laboratory showed that TBX2 was able to repress *α2(1) collagen* expression independent of a T-element, suggesting that TBX2 may be

acting as a co-repressor in these studies (Teng et al., 2007). Taken together, these contrasting results suggest that Tbx2 may regulate *type 1 collagen* genes by functioning as either co-activator or co-repressor depending on the cell context and/or the species.

1.3.3 TBX2 in cancer and the cell cycle

TBX2 is involved in normal breast development and studies have shown that its altered expression may play a role in the pathogenesis of breast cancer. *TBX2* was shown to be amplified and over-expressed in a subset of breast cancer cell lines and primary tumours. Using fluorescence *in situ* hybridisation, *TBX2* was shown to be amplified in BRCA1- and BRCA2-related breast tumours (Jacobs et al., 2000; Sinclair et al., 2002). Furthermore, *in situ* hybridisation of paraffin sections from sporadic and hereditary breast tumours showed that this amplification was associated with increased *TBX2* gene expression (Sinclair et al., 2002). Importantly, *TBX2* maps to chromosomal band 17q23, which is frequently amplified in many breast cancer cells (Law et al., 1995; Wu et al., 2001, Sinclair et al., 2002; Bärlund et al., 2000; Mahlamäki et al., 2002).

TBX2 has also been strongly implicated to play a role in the progression of pancreatic cancer, with fluorescence *in situ* hybridisation studies identifying *TBX2* to be amplified in 50% of pancreatic cancer cell lines tested as well as two independent studies showing TBX2 to be over-expressed in pancreatic tumours (Mahlamaki et al., 2002; Chen et al., 2008a; Duo et al., 2009). In both studies TBX2 over-expression in tumours correlated positively with the degree of tumour differentiation, higher clinical and pathological staging and distant metastasis (Chen et al., 2008a; Duo et al., 2009). In addition, *TBX2* was found to be over-expressed in tissue isolated from renal cell carcinomas and in primary melanomas and melanoma cell lines (Wang et al., 2004a; Vance et al., 2005). Interestingly, work in our laboratory has shown that TBX2 is down regulated in transformed human lung fibroblasts which suggests that both the up- and down-regulation of TBX2 is associated with cancer formation and that this may be cell-type dependent (Teng et al., 2007).

Tbx2 was also implicated in both the genesis of cancer and in cell cycle regulation in a senescence bypass screen aimed at identifying factors that could immortalise rapidly senescing *Bmi*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Jacobs et al., 2000). Senescence is the physiological program of irreversible growth arrest activated at the end of a cells lifespan or

in response to cellular stress. Bmi1 downregulates the genes transcribed from the *Cdkn2a* locus, *p16*^{INK4a} and *p19*^{ARF} (see section 1.4.2.1), and consequently *Bmi*$^{-/-}$ MEFs have been shown to rapidly undergo premature senescence (Jacobs et al., 1999). A retroviral cDNA library obtained from JEG3 human placental chorio-carcinoma cells was used to infect *Bmi*$^{-/-}$ MEFs and Tbx2 was identified as the primary factor able to confer immortalisation to these cells (Jacobs et al., 2000). The authors utilised chloramphenicol acetyltransferase (CAT) and luciferase reporter assays to show that bypassing of senescence was due to Tbx2s ability to repress *p19*^{ARF}, while p16^{INK4a} protein levels were comparable to that of senescent *Bmi*$^{-/-}$ MEFs. Furthermore, research conducted by another group showed that spontaneous immortalisation of MEFs following disruption of the *Scaffold attachment factor B1 (SAFB1)* gene, which encodes a nuclear protein involved in the stress response, was associated with increased Tbx2 and reduced p19 ^{ARF} levels (Dobrzycka et al., 2006).

Tbx2's role as an immortalising oncogene was further established in experiments which demonstrated that Tbx2 directly binds and represses the promoter of the cyclin dependent kinase inhibitor (CDKI, see section 1.4.2.1) *p21*, which led to senescence bypass of mouse ST.*Hdh*^{Q111} striatal cells (Prince et al., 2004). Moreover, expression of an inducible dnTbx2 in B16 mouse melanoma cells induced these cells to senesce and this was associated with an increase in *p21* expression (Vance et al., 2005).

TBX2 is not the only T-box factor that has been associated with the development of cancer. A large body of evidence supports a role for the transcriptional repressor TBX3, a close relative of TBX2, in tumourigenesis. For example, TBX3 is over-expressed in transformed human lung fibroblast cells and cell lines derived from melanomas, small cell lung and breast cancer as well as breast cancer tissue (Fan et al., 2004; Hoek et al., 2004; Turney et al., 2004; Yin et al., 2004; Yarosh et al., 2008). Furthermore, TBX3 was over-expressed in tumours isolated from the pancreas, liver and rat bladder and elevated levels of this protein were found in plasma samples of ovarian and breast cancer patients (Hansel et al., 2004; Ito et al., 2005; Lomnytska et al., 2005; Renard et al., 2007; Chen et al., 2009). Carlson et al. (2001) demonstrated that stable expression of Tbx3 immortalised MEFs and this was later shown to be through the ability of Tbx3 to repress the *p19*^{ARF} promoter via the same variant T-site recognised by TBX2 (Brummelkamp et al., 2002a; Lingbeek et al., 2002). Like Tbx2, Tbx3 can bind and repress the *p21* promoter and, in addition, Tbx3 can inhibit apoptosis and

cooperate with oncogenic Myc and Ras in cellular transformation assays (Carlson et al., 2002; Ito et al., 2005; Hoogaars et al., 2008).

Interestingly, Rodriguez et al. (2008) show that TBX3 can directly repress the promoter of the cell adhesion molecule *E-cadherin*. These authors utilised *in vitro* matrigel and cell-cell adhesion assays to demonstrate that this repression was functionally significant because it promoted melanoma invasiveness by reducing cell-cell adhesion. It is well established that reduced E-cadherin levels enables β-catenin to locate to the nucleus and transcriptionally activate growth promoting factors. Indeed, a study by Renard and colleagues (2007) found that over-expression of TBX3/Tbx3 in human and murine liver tumours was associated with activating mutations in *β-catenin* or an overactive β-catenin pathway. ChIP and luciferase reporter assays were utilised to show that *TBX3/Tbx3* is a direct target of β-catenin transactivation and siRNA-mediated silencing of TBX3 reduced the anchorage-independent growth of liver and colon carcinoma cells. Therefore these data suggest that β-catenin may regulate its own expression via the induction of *TBX3* which, through repression of *E-cadherin*, leads to increased invasiveness and increased nuclear localisation of its own activator. In support of a role for TBX3 in the latter stages of cancer progression, TBX3 was found to be over-expressed in metastatic pancreatic endocrine tumours compared to their non-metastatic counterparts (Hansel et al., 2004).

Taken together the above information suggests that TBX2 and TBX3 may contribute to the oncogenic process through inactivating key cell cycle checkpoint proteins. In addition this may suggest that under normal conditions these factors are important for regulating cell cycle progression. If this were the case then one might expect the protein levels of TBX2 and TBX3 to be regulated during the various phases of the cell cycle. Indeed, experiments by Bilican and Goding (2006) have shown that Tbx2 levels are low in G1, increase in mid-S-phase and persist at high levels through G2. Tbx2 levels do however undergo a dramatic reduction at the onset of mitosis. Unpublished data generated in our laboratory has shown similar results for TBX3 protein levels, but while TBX3 levels also increase in G2 they remain elevated during mitosis. These observations suggest that TBX2 and TBX3 levels are tightly regulated during the cell cycle, but indicate that these two closely related factors do play distinct roles.

The above data implicate TBX2 in the oncogenic process and this may be linked to its role in the cell cycle. The current study investigates this further and thus an understanding of the cell cycle and the transformation process is required.

1.4 *The cell cycle*

1.4.1 Phases of the cell cycle

The cell cycle is a complicated process that requires intricate regulation of numerous events which lead to the production of two identical daughter cells (reviewed by Schafer, 1998; Lebedeva et al., 2004; Tessema et al., 2004). It is broadly divided into two phases, interphase and M-phase (Figure 1.1). Interphase is further divided into G1, S and G2. S-phase is the stage at which the DNA is replicated and M-phase is where the successfully replicated chromosomes are divided equally between two daughter cells. G1 and G2, or the "gap" phases, are important for, among other roles, preparing the cell for entry into S- and M- phase respectively which ensures that these two processes occur with high fidelity. Once M-phase is completed, cells can either continue to cycle or they can enter into a state of terminal differentiation, quiescence (G_0) or senescence (Schafer, 1998; Blomen and Boonstra, 2007). Terminal differentiation is an irreversible state where cells are unable to divide and acquire specialised functions, as occurs in neurons for example. Quiescence is a temporary resting state that cells enter when conditions are not conducive for progressing into S-phase (Coller et al., 2006). Senescent cells on the other hand are cells which have irreversibly exited the cell cycle either at the end of their lifespan or in response to cellular stress.

1.4.1.1 Interphase

As stated above, the gap phases primarily prepare the cell for S- and M- phase (Schafer, 1998; Tessema et al., 2004). G1 is the interval between mitosis and DNA replication and is characterised by cell growth. In G1, cells have intricate mechanisms to determine whether they should arrest or progress to S-phase. Once committed to enter cell division, cells in late G1 synthesise proteins required for DNA synthesis, replicate organelles and increase in size. Duplication of the centrosome is initiated at the G1/S-phase transition and completed before mitosis. The two centrosomes (also known as the microtubule organizing centres) will act as poles of the spindle apparatus and are critical for successful mitosis. In S-phase the DNA is replicated resulting in two sister chromatids held together by their centromeres. Prior to entry

into M-phase, the cells enter G2, where the cell "proofreads" the DNA and prepares itself for mitosis.

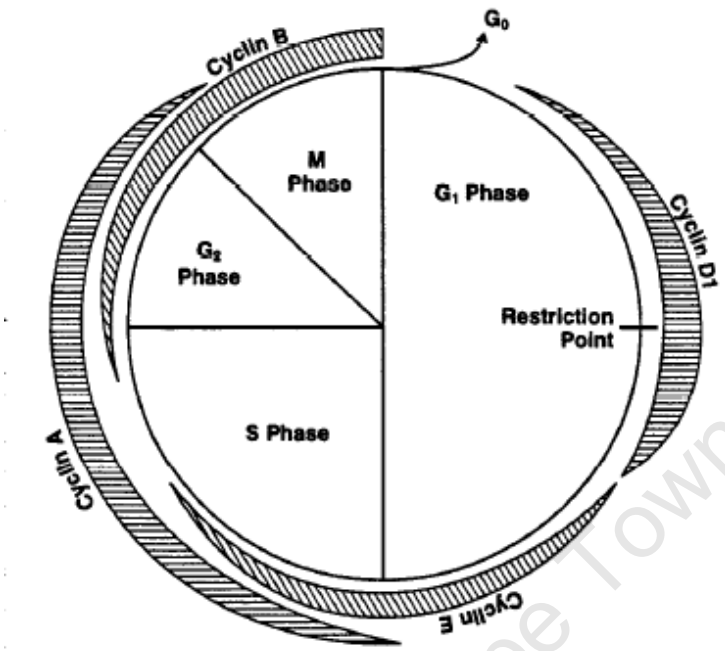

Figure 1.1. The eukaryotic cell cycle is divided into G1, S, and G2 (interphase) and M-phase. The levels of cyclins are regulated and fluctuate throughout the cell cycle, as shown above, and are responsible for the progression of the different phases of the cell cycle (from Schafer, 1998).

1.4.1.2 M-Phase

M-phase is generally divided into mitosis, where the duplicated sister chromatids are separated, and cytokinesis, where the cell divides to form two daughter cells (Fig 1.2) (reviewed by Nigg, 2001; Lebedava et al., 2004; Tessema et al., 2004). Mitosis itself can be divided into 5 phases: prophase, prometaphase, metaphase, anaphase and telophase.

During prophase the duplicated chromatin condenses into discrete chromosomes (Fig 1.2 (1)). Each of the duplicated centrosomes migrate to an opposite pole of the cell and the mitotic spindle, composed of microtubules, originates from and forms between, the two centrosomes. During prometaphase the nuclear envelope breaks down and the nucleolus disappears (Fig 1.2 (2)). Protein complexes, called kinetochores, assemble at the centromere of each chromatid and, due to the absence of the nuclear envelope, the mitotic spindle can access and bind to the centromeres at the kinetochores. This binding occurs by specific

kinetochore microtubules, which also attach to the centrosomes at either pole. At metaphase the attached chromatids align on the equatorial (metaphase) plate and a dynamic tension is established due to each sister chromatid being attached to opposite poles via the mitotic spindle (Fig 1.2 (3)). Once all chromatids are aligned, securin, the protein holding the sister chromatids together, is degraded and the kinetochore microtubules depolymerise, resulting in each chromatid being pulled to one of the centrosomes during anaphase (Fig 1.2 (4)). Telophase commences once the sister chromatids have successfully separated and arrived at each pole of the cell. During this phase the chromosomes decondense, the nuclear envelope begins to reform and the kinetochore microtubules disassemble. M-phase culminates with cytokinesis, where a contractile ring formed of actin filaments causes the pinching apart of the two identical daughter cells by a process called cleavage (Fig 1.2 (5)).

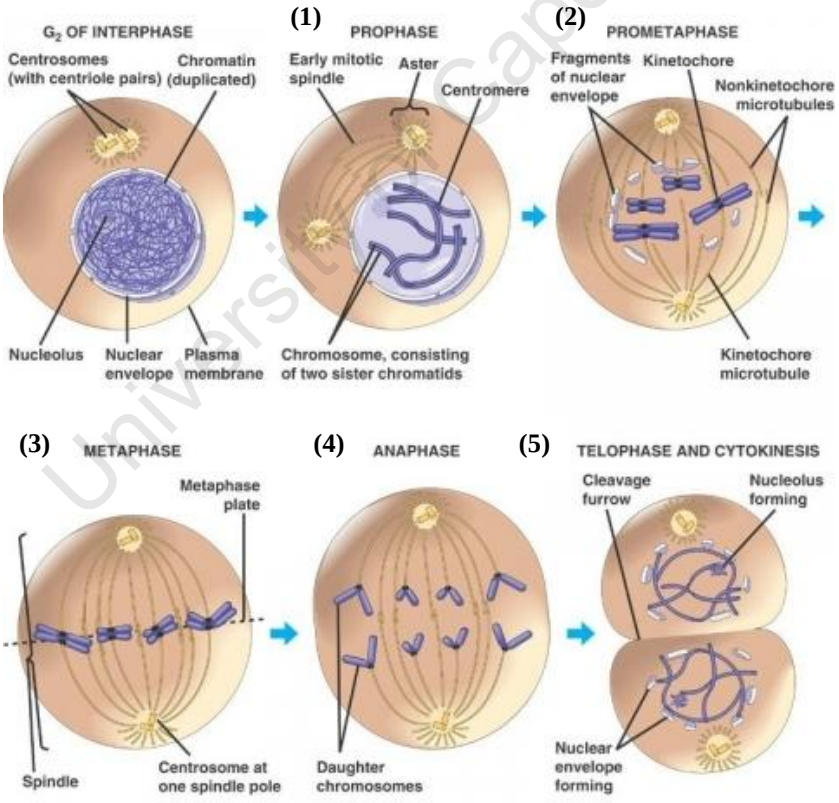

Figure 1.2. M-phase is a process of cell division which results in the production of two identical daughter cells from a single parent cell (royaleb.wordpress.com/2009/04/27/mitosis).

1.4.2 Control of the cell cycle

1.4.2.1 Cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors

The progression of the cell cycle from one phase to another is tightly regulated by the precise synthesis and destruction of a group of proteins called cyclins, whose name arises from the fact that their levels fluctuate throughout the different phases of the cell cycle (see fig. 1.1) (Evans et al., 1983; Murray and Kirschner, 1989, reviewed by Pines, 1995; Johnson and Walker, 1999; Vermeulen et al., 2003). When available, cyclins can bind and form complexes with their corresponding cyclin-dependent kinase (CDK) via a homologous domain common to all cyclins, called the cyclin box (Kobayashi et al., 1992). CDKs are serine/threonine protein kinases whose levels remain relatively constant throughout the cell cycle and they are only activated upon binding to their specific cyclin (Pines, 1995). Once activated, the cyclin/CDK complexes are able to bind to and phosphorylate a variety of substrates which, through either their activation or inactivation, drive the cell cycle from one phase to another (see fig.1.1).

Briefly, entry and progression through G1 into S-phase is regulated by the cyclin D/CDK4 or CDK6 and cyclin E/CDK2 complexes (Bates et al., 1994; Meyerson and Harlow, 1994**,** Johnson and Walker, 1999). These complexes phosphorylate and consequently inactivate the retinoblastoma protein (pRb) (Ohtsubo et al., 1995; Weinberg, 1995, Johnson and Walker, 1999; Vermeulen et al., 2003). Upon phosphorylation, pRb dissociates from the transcription factor E2F, enabling E2F to transcribe genes such as cyclin A and E, CDK1 and DNA polymerase α required for S-phase (Chellappan et al., 1991; Dynlacht et al., 1994; Tommasi and Pfeifer, 1995; Ohtani et al., 1995; Duronio et al., 1996; Izumi et al., 2000). While cyclin A/CDK2 drives progression through S-phase, both cyclin A/CDK1 (also called cdc2) and cyclin B/CDK1 play important roles in G2/M progression (Pagano et al., 1992). Degradation of cyclin A at mid-prophase enables the progression of the cell cycle into the later stages of mitosis (Furuno et al, 1999; den Elzen and Pines, 2001). Cyclin B/CDK1 also activates the anaphase promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase that is required for anaphase onset (Lahav-Baratz et al., 1995; Sudakin et al., 1995; Kraft et al., 2003). One of the functions of the APC/C is the degradation of cyclin B, a key event regulating mitotic exit, as failure of this leads to metaphase arrest (Murray et al., 1989; Glotzer et al., 1991; King et al., 1995; Sudakin et al., 1995).

Additional functions for cyclin/CDK complexes include DNA replication, DNA repair, differentiation and apoptosis and cyclin C/CDK8, cyclin T/CDK9, and cyclin H/CDK7 are components of the basal transcription apparatus (Peng et al., 1998). The activity of CDKs is also regulated by other mechanisms including phosphorylation, proteolysis due to ubiquitination and regulation by a family of proteins called cyclin dependent kinase inhibitors (CDKIs).

The CDKIs play an important role in controlling cell cycle progression through inactivating CDKs, either by binding to them directly or by binding to cyclin/CDK complexes (Johnson and Walker, 1999). Two families of CDKIs have been identified; the INK4 family and Cip/Kip family (Sherr and Roberts 1995, Johnson and Walker, 1999). Members of the INK4 family form complexes with the CDK prior to cyclin binding, thus preventing association with the specific cyclin (Carnero and Hannon 1998). Members of this family include p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d), and they specifically inactivate the G1 CDKs; CDK4 and 6. Interestingly, the *p16*^{INK4a} gene has an alternate reading frame that encodes another protein, p14^{ARF} (in humans) or p19^{ARF} (in mouse) (Sharpless and DePinho, 1999). Although p14^{ARF} has not been associated with CDK inhibition, it has been found to bind to, and sequester, the tumour suppressor protein p53 inhibitor MDM2 (Sherr, 1998; Sharpless and DePinho, 1999). p14^{ARF} thus functions as a regulator of the cell cycle by stabilising p53 protein levels.

Members of the Cip/Kip family, which include p21, p27 (Cip2) and p57 (Kip2), bind directly to cyclin/CDK complexes and in this way inhibit their activity (Polyak et al. 1994; Harper et al. 1995; Lee et al. 1995). The Cip/Kip CDKIs inhibit both the G1 cyclin/CDK complexes and the mitotic cyclin B/CDK1 (Hengst and Reed 1998). It is important to note that p21 can also form a complex with and block the proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase α. PCNA is the principal replicative DNA polymerase and this activity of p21 therefore leads to the arrest of DNA synthesis and allows for DNA repair (Flores-Rozas et al., 1994; Waga et al., 1994).

CDKIs themselves are regulated by various signalling pathways, for example p15, p21 and p27 are upregulated by the transforming growth factor β (TGF-β) pathway, a potent inhibitor of proliferation (Datto et al., 1995; Reynisdottir et al., 1995; Robson et al., 1999).

Importantly, p21 and $p14^{ARF}$ are transcriptionally activated by p53 in response to cellular stress which results in the triggering of checkpoints and hence cell cycle arrest (El-Deiry et al., 1993).

1.4.2.2 Checkpoints

The maintenance of the integrity of the genome and tight regulation of the cell cycle is essential in order to ensure successful cell division and the consequent health of the cell (reviewed by Elledge, 1996; Bartek and Lukas, 2001; Kastan and Bartek, 2004). Cells have therefore developed regulatory pathways, called checkpoints, which ensure that DNA replication and mitosis occur with fidelity. In response to cellular stress, these checkpoints either arrest the cell to enable repair processes to take place or, if the damage is irreparable, activate senescence or apoptotic pathways. The next section will describe the different checkpoints with particular reference to the cellular response to those triggered in response to DNA damage. The reason for this is firstly because it is believed that most stress ultimately results in damage to the DNA and secondly because this has relevance to this thesis.

- ***G1 checkpoints***

In response to suboptimal conditions, such as limited growth factors or high cell density, cells exit the cell cycle and enter a non-proliferating state in mid to late G1 (Bartek and Lukas, 2001). This is known as the restriction point and is a pRb-dependent checkpoint (Pardee 1974; Bartek and Lukas, 2001). A DNA damage checkpoint exists after the restriction point in G1 to monitor the integrity of the genome prior to DNA replication (Bartek and Lukas, 2001; Kastan and Bartek, 2004). For many years this checkpoint was thought to be activated exclusively through a p53-dependent pathway. However, the DNA damage response proteins Ataxia-Telangiectasia Mutated (ATM) and ATM and Rad 3-related (ATR) have now been shown to activate the G1 DNA damage checkpoint through both a p53-dependent and –independent cascade (Fig. 1.3).

The p53-independent cascade is initiated first when ATM, and possibly the related kinase ATR, phosphorylate, and thus activate, their downstream targets chk1 and chk2 (Costanzo et al., 2000; Mailand et al., 2000; Falck et al., 2001). These kinases in turn phosphorylate and inactivate cdc25A, a phosphatase responsible for activation of CDK2 in late G1 (Mailand et al., 2000). This results in cells not progressing into S-phase (Fig. 1.3).

Another p53-independent mechanism may be via the targeted degradation of cyclin D1 (Fig. 1.3). As mentioned previously, cyclin D/CDK4/6 is active in mid G1 whereas cyclin E/CDK2 drives the cell into S-phase. In response to genotoxic stress induced by ionising radiation, the cyclin D1/CDK4/6 complex is inactivated by targeted proteolysis of cyclin D1 via a 'destruction box' (Agami and Bernards, 2000). It has been proposed that this avails p21 to exclusively inhibit the cyclin E/CDK2 complex preventing entry into S-phase (Agami and Bernards, 2000).

The second G1 DNA damage response involves a delayed arrest mediated by p53 (Di Leonardo et al., 1994). This maintains the arrest initiated above to enable DNA damage repair and, if necessary, leads to the activation of apoptotic pathways (Kastan et al., 1991; Kuerbitz et al., 1992; Lowe et al., 1994). In this response p53 is activated by phosphorylation by ATM/ATR and chk1/2 (Fig. 1.3). While phosphorylation by ATM/ATR activates the transcriptional activity of p53, phosphorylation by chk1/2 results in p53 dissociating from MDM2 and consequently leads to an increase in p53 stability (Canman, et al., 1994; Chehab et al., 2000; Shieh et al., 2000). Once activated p53 transcriptionally regulates its target genes, including p21, to execute a cell cycle arrest and/or apoptosis (Waldman et al., 1995).

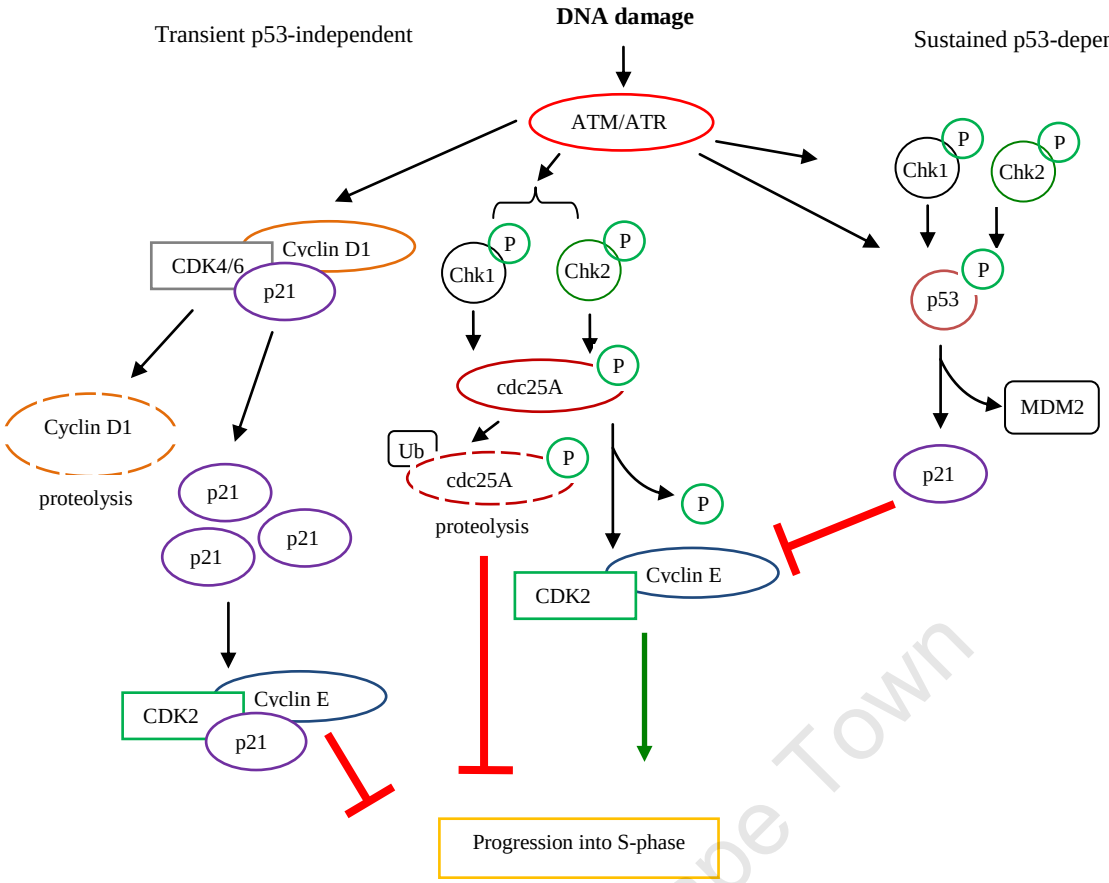

Figure 1.3. The G1 DNA damage checkpoint. In response to DNA damage a rapid, p53-independent cascade is activated either by the DNA damage response proteins Ataxia-Telangiectasia Mutated (ATM) and ATM and Rad 3-related (ATR) or via cyclin D degradation. ATM and ATR also activate p53, which initiates a second, more sustained arrest. All these pathways prevent progression into S-phase by inactivating the cyclin E/CDK2 complex (modified from Bartek and Lukas, 2001).

- ***Intra-S-phase checkpoint***

An arrest in S-phase can be triggered as a result of direct DNA damage or due to faulty DNA replication (Kastan and Bartek, 2004). A degree of overlap and cooperation must exist between the DNA damage and replication stress pathways since for example stalling of replication forks are elicited due to both these cellular responses. The S-phase, also known as the intra-S-phase, checkpoint enables the prevention of irreversible collapse of DNA replication forks, suppression of late origin firing and initiation of DNA repair (Kastan and Bartek, 2004). This checkpoint results in a transient arrest, compared to the more sustained arrest induced by the G1 and G2 checkpoints, presumably to prevent cells permanently arresting with an incomplete genome.

When DNA double strand breaks occur during S-phase, ATM is activated, which in turn can activate two different pathways which results in the inhibition of DNA replication and initiation of DNA repair (Fig. 1.4) (Kastan and Bartek, 2004). The pathway associated with inhibition of DNA replication involves activation of a chk2-cdc25A cascade similar to the p53-independent G1 DNA damage checkpoint (Falck et al., 2001). In the intra-S-phase checkpoint, however, a lack of CDK2 prevents preinitiation complexes from forming at the origins of replication (Falck et al., 2001). The intra-S-phase checkpoint pathway involving DNA repair requires the phosphorylation, and subsequent activation, of Nijmegen breakage syndrome 1 (NBS1) (Lim et al., 2000). NBS1 is a component of a protein complex containing Mre11 and Rad50 that is involved in initiating an S-phase arrest and in DNA double strand break repair. Active NBS1 leads to the recruitment of proteins, including the breast cancer 1 (BRCA1) and structural maintenance of chromosomes (SMC1) proteins, to the site of damage resulting in maintenance of the arrest, DNA repair and the insurance of chromosome integrity (Xu et al., 2001; Kitagawa et al., 2004). In addition, in response to single stranded DNA breaks, ATR is activated and this too can lead to the activation of similar pathways to that described above, but in a chk1 dependent manner (Fig. 1.4) (Shechter et al., 2004).

Evidence that the above two intra-S-phase pathways act in parallel comes from studies using ionising radiation which showed that interference with either one led to partial replication arrest. In addition, inhibition of both pathways entirely abolished the DNA replication arrest (Falck et al., 2002). Other factors, including Rb and p21, have also been implicated in the S-phase arrest but their exact role in this checkpoint and the degree of overlap with the above mentioned pathways remains to be elucidated.

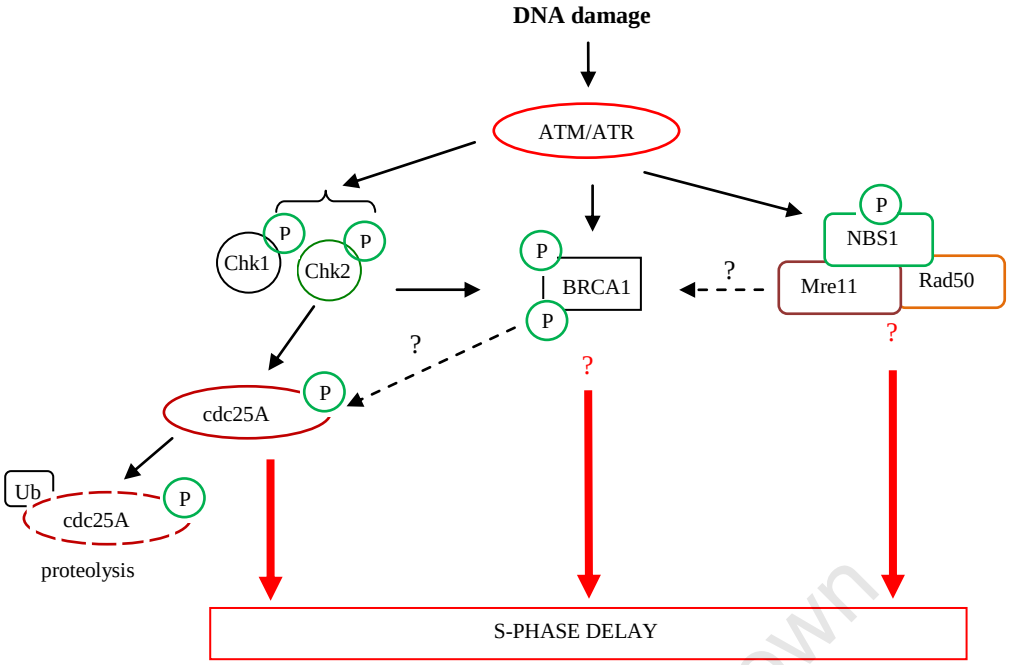

Figure 1.4. The pathways activated in the intra-S-phase checkpoint. Unlike the checkpoints activated in G1 and G2, the intra-S-phase checkpoint does not permanently arrest, but only delays S-phase in response to DNA damage. Upon activation, Ataxia-Telangiectasia Mutated (ATM) or ATM and Rad 3-related (ATR) initiate the chk2 or chk1-dependent degradation of cdc25A, which results in reduced DNA synthesis, or the activation of factors such as NBS1 and BRCA1 which are believed to contribute to DNA repair (modified from Kastan and Bartek, 2004).

- ***G2 checkpoint***

Cells that enter G2 with unrepaired DNA damage or cells that encounter damage during the G2 phase are arrested and prevented from entering mitosis. The pathways governing this checkpoint, however, are not well understood, but many factors, as described below, have been implicated (Kastan and Bartek, 2004). For example, in response to ionising radiation (IR), both ATM and ATR can be activated in G2 cells, resulting in the rapid phosphorylation of chk1/chk2 (Fig. 1.5) (Peng et al., 1997; Sanchez et al., 1997; Cliby et al., 1998; Matsuoka et al., 1998; Blasina et al., 1999). Both these kinases in turn, have been shown to phosphorylate cdc25C on Ser216 *in vitro*, which abolishes its ability to dephosphorylate and thus activate CDK1 (Peng et al., 1997; Sanchez et al., 1997; Matsuoka et al., 1998). The role chk1 and chk2 play in this checkpoint is illustrated by the observation that in response to IR, conditional chk1$^{-/-}$ or chk2$^{-/-}$ mouse embryonic stem cells fail to maintain a G2 arrest (Hirao et al., 2000; Liu et al., 2000). Furthermore, siRNA-mediated silencing of chk2 in human cells led to the abrogation of an oxidative stress signalling-induced G2 arrest (Kani et al., 2007). In

addition, chk2 is thought to maintain the G2 arrest through its ability to activate p53, which in turn activates p21, GADD45 and 14-3-3σ. This leads to the inactivation of the cyclin B/CDK1 complex and prevents entry into mitosis (Fig. 1.5) (Hermeking et al., 1997; Bunz et al., 1998; Chan et al., 1999; Wang et al., 1999; Zhan et al., 1999; Hirao et al., 2000; Jin et al., 2002).

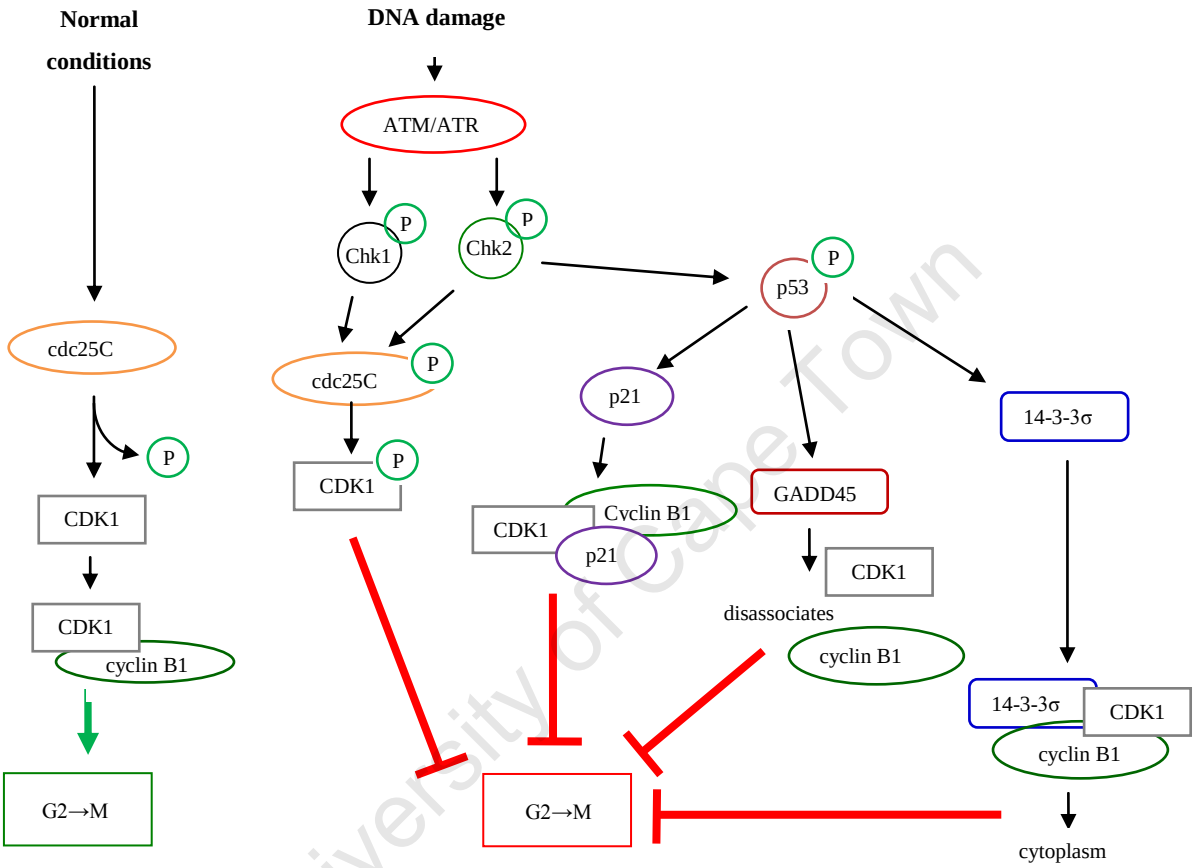

Figure 1.5. The pathways governing the G2 checkpoint. Under normal conditions cdc25C dephosphorylates and thus activates CDK1 which, in a complex with cyclin B1, is necessary for progression into M-phase. In response to damaged DNA activated ATM and ATR phosphorylate chk1 and chk2, which in turn abolish cdc25C-mediated activation of CDK1. In addition, chk2 is thought to maintain the G2 arrest through activation of p53 which can activate p21, GADD45 and 14-3-3σ. In all instances this leads to the inactivation of the cyclin B/CDK1 complex and thus prevents the cell progressing into mitosis.

- ***Spindle assembly and tetraploidy checkpoints***

During mitosis the equal segregation of sister chromatids to each spindle pole is very important to avoid the formation of daughter cells with an abnormal number of chromosomes

(aneuploidy). Aneuploidy has been shown to contribute to several developmental disorders, such as trisomy which can result in Down syndrome (Oliver et al., 2008). Furthermore, aneuploidy may also contribute to tumourigenesis (this will be discussed further in 1.5.4). The Spindle Assembly Checkpoint (SAC) ensures proper sister chromatid segregation by inhibiting the onset of anaphase until all chromosomes are properly aligned on the metaphase plate and are attached to kinetochore microtubules from each spindle pole (reviewed by Kops, 2008). While the basic order of events in the SAC pathway is well established, the precise molecular mechanisms regulating these events are not completely understood. Unattached kinetochores triggers the SAC by eliciting an inhibitory signal which results in the recruitment of a number of proteins, including Cenp-E, Mps1 and members of the mitotic arrest defective- (Mad1, Mad2 and Mad3) and budding uninhibited by benzimidazole- (Bub1, Bub2 and Bub3) families (Rieder et al., 1994; 1995; Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). These SAC proteins were initially identified in the yeast *Saccharomyces cerevisiae*, and homologues of all these factors have since been identified in mammals. While the identity of the inhibitory signal is still being elucidated, it has been established that upon arrest Mad2- and Bub2- dependent- pathways are activated.

The Mad2-dependent pathway, which involves Mad1, Mad2, Bub1, BubR1 (Mad3 mammalian homologue) and Bub3, prevents premature separation of sister chromatids and is activated by kinetochores that are not properly attached to spindle microtubules (Fig. 1.6) (Li et al., 1996; Taylor et al., 1998). In addition, Mad2 and BubR1 appear to play an important role as effectors of this checkpoint response since immunoprecipitation assays show that they bind, and probably inhibit, CDC20, a component of the APC/C (Fang et al., 1998; Hwang et al., 1998; Kim et al., 1998; Hardwick et al., 2000; Wu et al., 2000). This resulting inhibition of APC/C activity inhibits anaphase onset by preventing the degradation of proteins including cyclin B and securin, as discussed earlier (Sudakin et al., 1995; Cohen-Fix et al., 1996).

The Bub2-dependent response of the SAC, which prevents mitotic exit, is believed to be regulated by the Bub2 protein in association with another factor, Bfa1 (Alexandru et al., 1999; Li, 1999). Both these factors have been shown to associate with the budding yeast spindle pole body (the mammalian centrosome), and it is proposed that mitotic exit and cytokinesis are blocked through inhibition of centrosome migration (Fraschini et al., 1999; Li, 1999; Pereira et al., 2000; Lee et al., 2001).

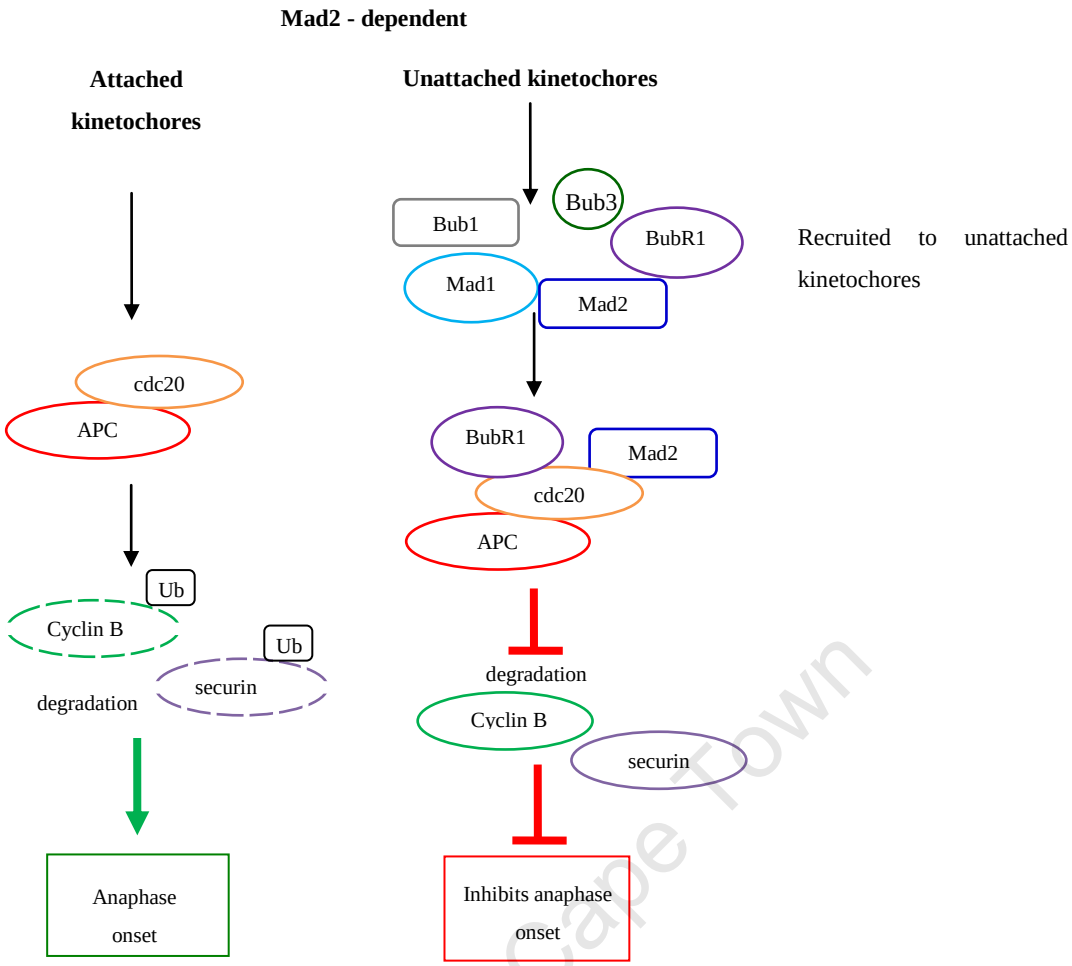

Figure 1.6. The Mad2-dependent pathway of the spindle assembly checkpoint (SAC). In response to unattached kinetochores the APC/cdc20 complex ubiquitinates proteins, such as cyclin B and securin, that promote anaphase onset. Unattached kinetochores, however, recruit proteins including Mad2 and BubR1 which bind and most probably inhibit cdc20, thus preventing the onset of anapahse.

In addition to the SAC, cells also have a postmitotic G1 checkpoint to prevent the propagation of aneuploid cells. In normal cells, prolonged activation of the SAC can result in cells undergoing mitotic slippage. In this instance cells exit mitosis without having undergone successful chromosome segregation and cytokinesis, thus entering G1 with 4N DNA content (Minn et al., 1996). The resulting tetraploid cell is prevented from re-entering the cell cycle by an arrest in G1 at what is now known as the tetraploidy checkpoint (Margolis et al., 2003). Initial experiments investigating the tetraploidy checkpoint utilised microtubule poisons such as nocodazole to induce tetraploidy leading to the misconception that this postmitotic G1 arrest may be activated in response to spindle defects that trigger SAC (Di Leonardo et al., 1997; Lanni and Jacks, 1998). A series of subsequent experiments using dihydrocytochalasin, a drug that inhibits cleavage while spindle function and chromatid segregation remain

normal, demonstrated that it is the tetraploidy state itself that induces this G1 arrest (Andreassen et al., 2001).

Interestingly, work by Hinchcliffe et al. (2001) indicates that centrosomes may also play an important role in activating the tetraploidy checkpoint. They show that cells in which the centrosome had been surgically removed during S-phase proceeded through G2 and M-phase, but were arrested in G1. The implication of these results can be appreciated if one considers that cells that undergo a defective cytokinesis enter G1 with not only double the DNA content, but also double the normal number of G1 centrosomes. Furthermore, centrosome replication is intricately linked to the cell cycle and subsequent replication of tetraploid cells result in centrosome replication and the establishment of a multipolar spindle (Andreassen et al., 2001). It is therefore conceivable that centrosomes may elicit a signal notifying the cell of abnormal centrosome number in order to prevent aneuploid cells from arising.

The tetraploidy checkpoint is mediated primarily by p53 and pRb (see fig. 1.7) as demonstrated in experiments using human fibroblasts expressing either the HPV-16 E6 or E7 oncoproteins, which inactivate p53 and pRb respectively (Khan and Wahl, 1998). In these experiments, following mitotic failure these cells did not arrest in G1 with 4N DNA content (which was the case for their WT counterparts), but rather continued to cycle as polyploid cells (Khan and Wahl, 1998). p53's involvement in the tetraploidy checkpoint has been demonstrated to be through the activation of p21 and pRb (Khan and Wahl, 1998). Importantly, expression of a dominant negative E2F construct in pRb-deficient cells abrogates the effect of loss of pRb activity following nocodazole treatment (Srinivasan et al., 2007). These results confirm that in the tetraploidy checkpoint, pRb functions by its well established ability to bind and sequester E2F. The CDKI, $p16^{INK4a}$, has also been implicated in the tetraploidy checkpoint arrest because of observations that $p16^{INK4a}$ deficient cells prevented from undergoing a successful mitosis have an increase in ploidy (Khan and Wahl, 1998). However, $p16^{INK4a}$ is thought to function through an unidentified, p53-independent pathway in this checkpoint (Khan and Wahl, 1998). It is important to note that even though the SAC and tetraploidy checkpoints are distinct, a degree of overlap between these two checkpoints has been demonstrated. Results from experiments using HCT116 cells (WT p53 and an intact SAC) and their $p53^{-/-}$ or SAC-compromised counterparts show that a prolonged

SAC arrest is required for proper post mitotic G1 arrest in order to prevent subsequent endoreduplication (Vogel et al., 2004).

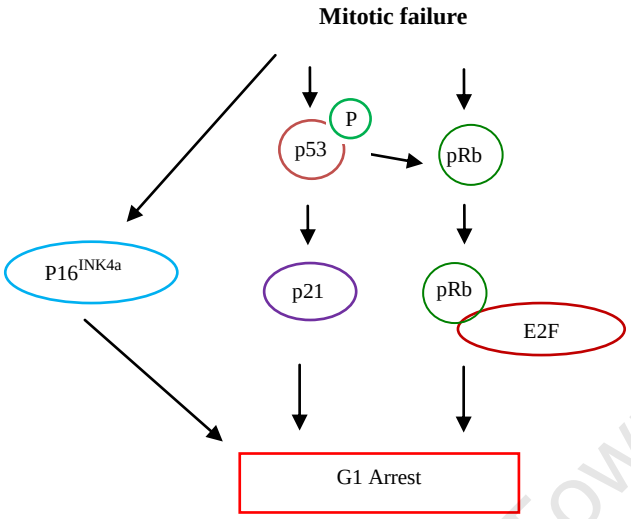

Figure 1.7. The tetraploidy checkpoint. Cells that undergo mitotic failure and thus enter G1 with 4N DNA content activate the G1 tetraploidy checkpoint. This checkpoint can be activated via p53- and/or pRb-dependent pathways or in a $p16^{INK4a}$- dependent manner.

From the above studies, it is evident that cells have established a complex network of checkpoints that function to prevent polyploid cells from cycling. Bypass of these checkpoints results in subsequent replication of the tetraploid cell, which lead to cells with chromosomal instability and aneuploidy which are associated with congenital diseases and are a common characteristic of cancer cells. This will be discussed in the next section.

1.5 *Cancer*

Normal, untransformed cells require mitogenic signals and favourable growth conditions in order to replicate. These signals and conditions are monitored by regulatory pathways which prevent inappropriate cell proliferation. Transformed cells, on the other hand, are able to bypass these regulatory pathways so that they are able to divide under unfavourable proliferative conditions. The transformation of normal cells into highly malignant derivatives is a multistep process which includes cell immortalisation, transformation and metastasis, with each of these steps being a manifestation of genetic alterations (as reviewed by Hanahan and Weinberg, 2000; Rakoff-Nahoum 2006; Croce, 2008; Mees et al., 2009). Although the

specific genetic changes that occur among cancer cells vary, it is generally agreed that there are six characteristic phenotypic properties that many metastatic cells acquire during the transformation process. These are (1) unlimited replicative ability (immortalisation), (2) reduced dependence on growth signals, (3) unresponsiveness to growth-inhibitory signals, (4) evasion of apoptosis, (5) induced angiogenesis and (6) invasive and metastatic potential (Hanahan and Weinberg, 2000).

1.5.1 Immortalisation

Immortalisation, the first step in cancer progression, is defined by the ability of a cell to divide infinitely. It is believed to facilitate cancer progression because unlimited cell divisions enable the acquisition of additional genetic alterations (Smith and Pereira-Smith, 1996). Cells have therefore devised a mechanism to ensure that they undergo a limited number of divisions, after which they irreversibly exit the cell cycle through a process known as replicative senescence (or M1 stage senescence) (Hayflick and Moorhead, 1961, reviewed by Shay and Wright, 2005). This process occurs in response to progressive shortening of telomeres with each round of cell division (Wright and Shay, 1992; Shay and Wright, 2005).

Telomeres are repetitive genetic elements located at the ends of chromosomes which protect the genomic DNA from being eroded (reviewed by Shay and Wright, 2005; Bailey and Murnane, 2006). Normally the single-stranded nucleotide overhang at the ends of the telomere invades upstream double stranded telomeric DNA and, together with associated telomere-binding proteins, forms a structure known as the t-loop (Fig. 1.6) (Griffith et al., 1999; Bailey and Murnane, 2006). When telomeres reach a critical length the telomere ends become exposed and activate, what is believed to be, a DNA damage-type signal. The mechanism by which this signal becomes activated is not completely understood, but is known to involve p53, which activates p21 and pRb to initiate an arrest (Shay et al., 1991; Beauséjour et al., 2003). Furthermore, $p16^{INK4a}$ is required for senescence and is believed to be particularly important for maintenance of this senescence arrest (Shay et al., 1991; Alcorta et al., 1996). Its importance is illustrated by observations that patients with familial melanoma display mutations in the CDKN2A locus which leads to loss of $p16^{INK4a}$ function (Hussussian et al., 1994; Sviderskaya et al., 2003). Furthermore reduced $p16^{INK4a}$ expression was observed in approximately 50% of breast cancers analysed in a study by Brenner et al.

(1996) and a number of spontaneously- and benzo(a)pyrene- immortalised breast epithelial cell lines have mutated $p16^{INK4a}$ (Brenner and Aldaz, 1995; Brenner et al., 1996).

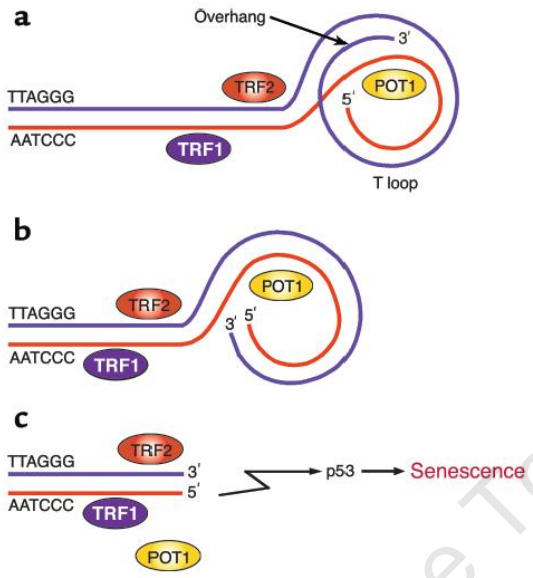

Figure 1.8. Telomere uncapping at senescence. (a) The structure of the t-loop. (b) Shortening of telomeres results in the single stranded portion being eroded. (c) Uncapping of telomeres leads to the activation of senescence pathways (from Ben-Porath and Weinberg, 2004).

Abrogation of the pathways responsible for replicative senescence, through the action of viral oncoproteins or other events, enables cells to bypass M1 and continue to divide (Shay and Wright, 2005). This leads to continued shortening of telomeres, finally resulting in crisis (M2 stage senescence) which is characterised by 'uncapped' chromosome ends, resulting in mitotic catastrophe and apoptosis (this will be described in greater detail in section 1.5.4).

Immortalisation therefore requires cells to bypass both M1 and M2. One mechanism by which this can be achieved is through the expression of telomerase, the enzyme responsible for extending telomeres by adding hexanucleotide repeats onto the ends of chromosomes (Counter et al., 1992; Shay and Wright, 2005). Indeed, approximately 90% of human cancers express telomerase activity and ectopic expression of this enzyme can immortalise normal presenescent cells (Feng et al., 1995; Shay and Bacchetti, 1997; Bodnar et al., 1998). M2 can also be bypassed through recombination or fusion between sister chromatids which prevents the cell detecting critically short telomeres (Shay and Wright, 2005) (the consequences of this is described in section 1.5.4).

It is interesting to note that uncapped telomeres have also been shown to associate with DNA damage response proteins. For example, experiments where either of the ATM/ATR and Chk1/Chk2 DNA damage response pathways were inactivated resulted in senescent human BJ cells re-entering the cell cycle (d'Adda di Fagagna et al., 2003). Furthermore, certain DNA damage response proteins have been found to have important functions in normal telomere maintenance (Nugent et al., 1998; Surrallés et al., 2004). Thus replicative senescence and the DNA damage response pathways appear to be intricately linked.

Accelerated senescence (also known as stress- or oncogene-induced senescence) differs from replicative senescence because it is induced prematurely by stresses such as DNA damage, environmental stress, cellular- and viral oncoproteins (Di Leonardo et al., 1994; Serrano et al., 1997). There is, however, a degree of similarity between both forms of senescence. For example, cells undergoing either replicative or accelerated senescence activate similar pathways, involving p53 and pRb and display similar characteristics, including a flattened morphology and expression of senescence associated β-galactosidase (Shay et al., 1991; Dimri et al., 1995; Braig and Schmitt, 2006). However, expression of the catalytic subunit of telomerase does not abrogate accelerated senescence which suggests that it is a process independent from replicative senescence (Gorbunova et al., 2002). It is worth noting that replicative and accelerated senescence are now recognised as important anti-cancer mechanisms that arrest the proliferation of aged, damaged or stressed cells that may be at risk for malignant transformation.

1.5.2 Transformation

Transformation is described as the progression of immortal cells to a more cancerous phenotype and occurs through the acquisition of multiple genetic mutations. This is due, in part, to transformed cells developing mechanism(s) which enable them to escape the constraints which would otherwise limit the proliferation of normal cells, such as reduced mitogenic stimuli, loss of cell-cell contact and anchorage (substrate) independence (Hanahan and Weinberg, 2000; Croce, 2008). For example, transformed cells can upregulate the expression of growth factor receptors, making these cells hyperesponsive to weak mitogenic signals in the environment that would not usually stimulate proliferation (Hanahan and Weinberg, 2000). Additionally transformed cells can display self-sufficiency in growth

signals by producing their own growth factors and/or other components of proliferation signalling pathways making them independent of external stimuli. An example that is common to many cancers is the constitutive activation of the Ras family of GTP-binding proteins or its' downstream effector proteins such as c-Myc. The Ras signalling pathway results in the induction of cyclin D and the activation of CDK2 and CDK4, thus driving cells inappropriately through G0/G1 and into S-phase (Dobrowolski et al., 1994; Aktas et al., 1997).

Normal cells are sensitive to cell-cell and cell-matrix adhesion and will not proliferate in the absence of a substrate or in a high cell density environment. This reduces aberrant cell motility and proliferation and is important in the development and maintenance of tissues (Gumbiner, 1996; Conacci-Sorrell et al., 2002). Transformed cells decrease their sensitivity to contact and substrate inhibition, in part, through the downregulation of cell adhesion molecules (CAMs) which are transmembrane glycoproteins located on the cell surface that facilitate cell–cell and cell–extracellular matrix (ECM) adhesion (Hirohashi and Kanai, 2003; Okegawa et al., 2004). Several large CAM superfamilies have been identified, including the immunoglobulin (Ig)-like CAMs, cadherins, selectins, and integrins. Due to the extensive information available on the CAM superfamily, the role of E-cadherin and integrins in transformation will be discussed to illustrate the important role that these factors play in this process.

E-cadherin forms the functional component of adherens junctions between epithelial cells and thus facilitates strong intercellular adhesions (Shiozaki et al., 1998; Gumbiner, 2000). Following cell-cell adhesion, the E-cadherin intracellular domain binds to a complex of α, β and γ catenin molecules. This complex in turn interacts with the underlying actin cytoskeleton, which assists in stabilising these cell-cell adherens junctions. Catenins also play an important role in linking cell-cell adhesion to cell proliferation because it forms part of the signalling pathway transducing signals from the cell surface (Gumbiner, 2000; Conacci-Sorrell et al., 2002). For example, Motti et al. (2005) demonstrated that in high cell density environments, E-cadherin/β-catenin signalling in cells that display contact inhibition leads to the upregulation of $p27^{kip1}$ which arrests cells in G1. This is due to consequent inactivation of cyclin E/CDK2 and dephosphorylation, and thus activation, of pRb (Motti et al., 2005). It is important to note that β-catenin can also transactivate factors that stimulate proliferation such

as cyclin D and myc and that the levels of cadherin play an important role in determining whether β-catenin regulates proliferation positively or negatively (He et al., 1998; Shtutman et al., 1999). Increased cadherin expression leads to the sequestration of β-catenin from the nucleus into adherens junctions and thus prevents it from transcriptionally activating its target genes (Conacci-Sorrell et al., 2002). Reduced E-cadherin expression in transformed cells therefore contributes to both the loss of cell-cell adhesion as well as deregulated cell proliferation. This is further underscored by the ability of neutralising E-cadherin antibodies to stimulate proliferation when introduced into contact-inhibited, G1 arrested cells (St. Croix et al., 1998; Motti et al., 2005). Finally, the important role that E-cadherin and β-catenin play in preventing cells acquiring transforming ability, is highlighted by the observation that they are inactivated in many cancers and germline mutations in the gene for E-cadherin, *CDH1*, predisposes individuals to hereditary gastric cancer (Conacci-Sorrell et al., 2002; Hirohashi and Kanai, 2003).

The integrin family is composed of at least 18 α and 8 β subunits that, through heterodimeric combinations, comprise at least 24 different members (Lu et al., 2008). Integrins, like other CAMs, facilitate direct interaction between cells and are involved in transducing signals from the cell surface. Integrin signalling has been shown to play an important role in varying processes such as cell migration, proliferation, differentiation and survival (Varner and Cheresh, 1996). In addition, integrins bind components of the ECM, such as fibronectin, laminin and collagen and thus also play an important role in anchoring cells to the ECM (Chioni and Grose, 2008; Lu et al., 2008). Interestingly, different integrin family members have been shown to have opposing effects on transformation. For example, loss of integrin α5β1 and α2β1expression was associated with an enhanced tumourigenic phenotype in Chinese hamster ovary and breast epithelial cells respectively (Varner and Cheresh, 1996). Reduced integrin αv expression however results in reduced tumourigenicity of melanoma and breast cancer cells (Varner and Cheresh, 1996; Chen et al., 2008b).

Most normal cells undergo terminal differentiation when they are fully specialised to perform specific functions and this process is accompanied by a loss of proliferative ability. Transformed cells, however, often display a partially or undifferentiated phenotype which mimics proliferating immature cells (Sell, 1993). Indeed, less differentiated tumours frequently represent a more advanced and more aggressive cancer. The basis for this undifferentiated state is currently under much discussion (as reviewed by Bapat, 2007; Wu,

2008). One school of thought proposes that undifferentiated cancer cells arise from mature cells which undergo dedifferentiation through the deregulation of signalling pathways. Another school of thought however proposes that the origin of undifferentiated cancer cells is mutated tissue stem cells which are able to proliferate and which forms the core of tumours (reviewed by Wicha et al., 2006). It is important to note that there is substantial evidence to support both theories which may suggest that they are both possible.

Finally, transformed cells are also able to proliferate in unfavourable conditions or in the presence of cellular damage due to their ability to evade apoptosis (Hanahan and Weinberg, 2000; Croce, 2008). This ability is associated with a decrease in pro-apoptotic factors or an increase in anti-apoptotic factors. For example many cancer cells upregulate the anti-apoptotic factor Bcl-2 and the majority of malignant breast cancer specimens tested in a study by Bargou et al (1995) had reduced expression of the pro-apoptotic factor Bax (Sartorius and Krammer, 2001). Indeed, patients that display decreased Bax levels are generally unresponsive to therapy and have reduced overall survival (Krajewski et al., 1995).

While a combination of the above mechanisms enables transformed cells to form tumours *in vivo*, the tumour size is limited without a vascular supply. Indeed, to progress from the transformed phenotype to a malignant phenotype cancer cells must acquire the ability to induce angiogenesis (Hanahan and Weinberg, 2000). This is achieved through the upregulated expression of angiogenesis-initiating factors, such as vascular endothelial growth factor, acidic and basic fibroblast growth factors and angiopoietin-2, which bind to receptors on the surface of nearby endothelial cells (Hanahan and Weinberg, 2000; Mazitschek and Giannis, 2004). Tumours can also develop their own vasculature by down-regulating inhibitors of angiogenesis, such as thrombospondin-1 or β-interferon (Hanahan and Weinberg, 2000). In addition to facilitating growth of the primary tumour, the new blood vessels provide a route through which cancer cells can migrate to distant organs (Aznavoorian et al., 1992) by a process referred to as metastasis which will be further discussed below.

1.5.3 Metastasis

In order for cancer cells to metastasise they must be able to invade adjacent tissues, survive in circulation, and establish a new tumour mass at a distant site where it is then called a secondary or metastatic tumour (Fig. 1.7) (Aznavoorian et al., 1992; Hanahan and Weinberg,

2000; Joyce and Pollard, 2009). The secondary tumour is a hybrid of the primary tumour cells and the invaded tissue and must retain key features of transformed cells in order to form a successful metastatic growth (Hanahan and Weinberg, 2000; Joyce and Pollard, 2009). The molecular mechanisms involved in invasion and metastases are not completely understood, but it is evident that in order for tumour cells to acquire this ability, additional genetic alterations are required (Aznavoorian et al., 1992). The defining feature of a metastatic cell is the ability to invade neighbouring tissue by firstly penetrating the epithelial basement membrane and then invading the blood vessels by a process known as intravasation (Aznavoorian et al., 1992). Extracellular proteases are responsible for the degradation and penetration of the basement membrane. These proteases also cleave cell-adhesion molecules, such as E-cadherin, which reduces cell-cell contact and thus assists in increasing cell motility (Joyce and Pollard, 2009). Whereas primary tumours often upregulate expression of proteases, the major producers of these enzymes are recruited adjacent normal cells such as stromal and bone marrow derived cells (Hanahan and Weinberg, 2000; Joyce and Pollard, 2009). It is important to note that degradation and invasion of the basement membrane is also necessary for tumour cells to exit blood vessels and enter distant organs at the secondary tumour site by a process known as extravasation (Aznavoorian et al., 1992). The direct role that proteases play in facilitating invasion is demonstrated in experiments where the ectopic expression of human matrix metalloprotease-9 in poorly metastatic cells resulted in an increase in invasiveness which was directly proportional to MMP-9 activity (Aalinkeel et al., 2004). Indeed, the pharmacological inhibition of some members of these protease families leads to a marked reduction in cancer cell invasion (Joyce and Pollard, 2009).

As mentioned previously, invasion and migration into the surrounding tissue also requires reduced cell-cell adhesion and increased motility and hence involves CAMs (Aznavoorian et al., 1992; Conacci-Sorrell et al., 2002). While there is a wealth of information available to illustrate the importance of CAMs in these processes, the involvement of integrins is particularly interesting. Integrins can facilitate invasion and metastases due to changes in integrin expression and because different integrin subtypes have distinct substrate preferences it enables adaptation of the invasive cancer cell to the changing microenvironment that it encounters during its journey (Varner and Cheresh, 1996; Hanahan and Weinberg, 2000).

As has been suggested above the multistep progression of a normal cell to a tumour cell requires an elevated mutation rate in the genome. This provides a pool of mutations in the cell, some of which provide a selective growth advantage. The mechanism by which this increased mutation rate arises will be discussed next.

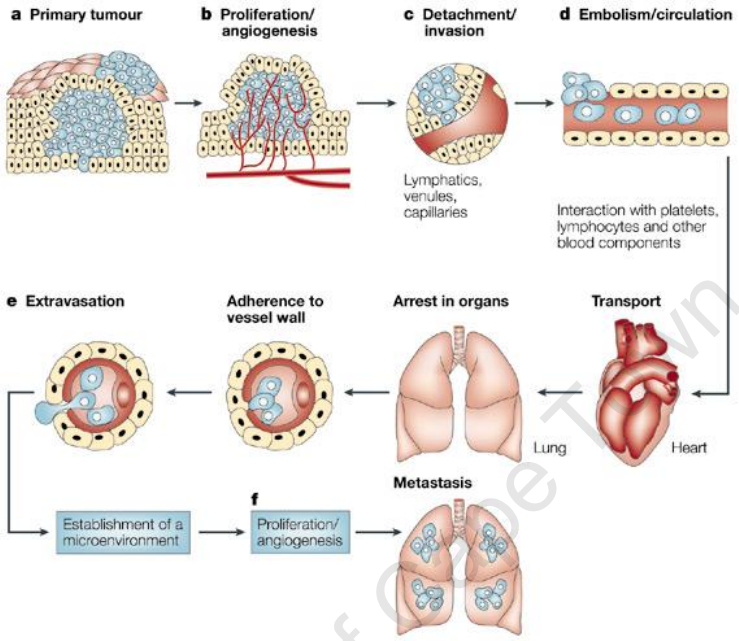

Figure 1.9. The process of transformation and metastases. (a) Following immortalisation, the cells of the primary tumour acquire the ability to proliferate in an uncontrolled fashion forming the primary tumour. (b) The induction of angiogenesis enables the primary tumour to increase in size. (c) Reduced adherence and increased invasive potential enables cancer cells to invade the surrounding stroma and vascular and/or lymphatic system. (d) Cells able to survive in the circulatory system migrate to distant sites in the body. (e) Invasive ability enables cancer cells to extravasate and (f) establish secondary tumours (from Fidler, 2003).

1.5.4 Genomic instability in cancer

Genomic instability is the continuous acquisition of genetic alterations, which can occur at a nucleotide level, involving mutations in individual genes and microsatellite repeat sequences, or at a whole chromosome level, involving the gain, loss and structural rearrangement of chromosomes (Anderson, 2001; Gisselsson, 2001; Raptis and Bapat, 2006; Perera and Bapat, 2007). At the nucleotide level genomic instability can arise through a number of mechanisms, one being elevated levels of DNA damage due to carcinogens which overwhelm the DNA damage response (Anderson, 2001, Mills et al., 2003; Kastan and Bartek, 2004). This can confer a growth advantage to cells and indeed the majority of cancers display genetic

alterations in genes involved in growth regulation, cell cycle progression and arrest (Ratis and Bapat, 2006). Examples include point mutations in *K-ras* resulting in its inappropriate activation in approximately 39% of human colorectal cancers analysed as well as 45-50% of all human cancers harbouring inactivating point mutations in the *p53* gene (Breivik et al., 1994; Soussi et al., 2000; Pihan and Doxsey, 2003). Moreover specific mutations resulting in the inactivation of *pRb* are associated with retinoblastoma, the most common childhood malignant ocular cancer (Benedict et al., 1990).

Instability at a nucleotide level may also result from defective DNA damage pathways that are unable to repair normal damage that the cell encounters (Anderson, 2001). This includes pathways involved in base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) (Charames and Bapat, 2003; Barnes and Lindahl, 2004; Kunkel and Erie, 2005; Leibeling et al., 2006; Perera and Bapat, 2007). The contribution of these defective pathways to tumourigenesis is illustrated by syndromes that are characterised by mutations in DNA repair genes and increased susceptibility to cancer (Heinen et al., 2002; Perera and Bapat, 2007). For example, germline mutations resulting in defective MMR are associated with hereditary non-polyposis colorectal cancer, and these individuals are at a greater risk of developing other cancers such as endometrial and ovarian cancer (Aaltonen et al., 1994; Aarnio et al., 1999; Heinen et al., 2002). Moreover, mutations in NER genes are synonymous with the disease Xeroderma Pigmentosum, where individuals are predisposed to skin cancer (Hoeijmakers, 1994; Heinen et al., 2002).

Genomic instability involving whole chromosomes can result in the continuous acquisition of structural and numerical genomic abnormalities which is then referred to as chromosomal instability (CIN) (Gisselsson, 2001; Pihan and Doxsey 2003; Storchova and Kuffer, 2008). In general, CIN arises due to errors in DNA damage repair, cell-cycle checkpoint regulation and chromosomal segregation. Faults in these processes lead to mitotic defects, such as anaphase bridges, multipolar mitoses, lagging chromosomes and micronuclei (Gisselsson and Höglund, 2005) and each of these is discussed below because it has relevance for this thesis.

- ***Anaphase bridges and multipolar mitoses***

Anaphase bridges are stretches of chromatin which connect the two separating chromosome masses during anaphase (McClintock, 1941). As first described by Barbara McClintock in

1941, anaphase bridges form as a result of abnormal chromosomal fusions due to the presence of 'free' or 'uncapped' chromosome ends (Gisselsson, 2001; Storchova and Pellman, 2004). An 'uncapped' chromosome end can occur due to loss of telomere function, which can be a consequence of malfunctioning telomere-associated proteins required for end-capping, or alterations that lead to the loss of sufficient repeat sequences necessary to maintain proper telomere structure (Lo et al., 2002; Maser and DePinho, 2002; Gisselsson, 2003; Bailey and Murnane, 2006). Once the telomeres are shortened past a critical length, the t-loop can no longer form and the proteins that protect the telomere ends detach.

These free chromosome ends now appear as DNA double strand breaks and may fuse to other chromosome ends with short telomeres. Alternatively, the 'uncapped' ends can result from DNA double strand breaks (Gisselsson, 2003; Mills et al., 2003; Pfeiffer et al., 2004; Bailey and Murnane, 2006). While the majority of double strand breaks generated in a cell are repaired correctly, with the original broken ends being ligated back together, misligation can occur where the cellular machinery will incorrectly fuse chromosome ends in an attempt to restore the original chromosome structure (Pfeiffer et al., 2004). For example, non-related chromosomes may fuse to generate dicentric chromosomes (Fig. 1.8) (Maser and DePinho, 2002; Storchova and Pellman, 2004; Gisselsson and Höglund, 2005) or, if both ends of one chromosome become free, they can undergo arm-to-arm fusion and form a ring chromosome (Fig. 1.8) (Gisselsson, 2001).

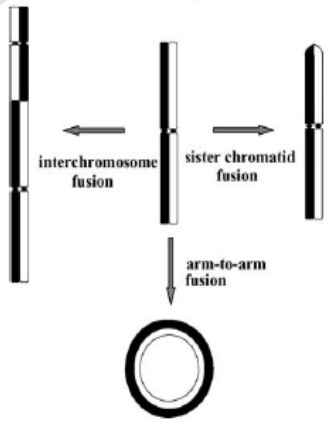

Figure 1.10. The consequences of free DNA ends. Telomere dysfunction or DNA double strand breaks enable unprotected chromosome ends to recombine. Interchromosome fusion can result in dicentric derivatives, and arm-to-arm fusion can lead to ring chromosomes. All of the cases shown above will form anaphase bridges during mitosis (from Gisselsson and Höglund, 2005).

Dicentric and ring chromosomes, together with sister chromatid fusion, result in chromosome structures that contain two centromeres and so can attach to both spindle poles (Gisselsson, 2001; Gisselsson and Höglund, 2005). This results in the fused chromosome being pulled towards opposite poles simultaneously during anaphase and the consequent formation of a bridge. Anaphase bridges can break, generating unstable ends which will again undergo fusions in the following interphase, resulting in continuous Breakage-Fusion-Bridge (BFB) cycles (Fig. 1.9) (McClintock, 1941; Gisselsson, 2001; Desmaze et al., 2003; Stewenius et al., 2005; Bailey and Murnane, 2006). The breaks in the chromosomes that result during BFB cycles are generally not at the initial site of fusion, thus continuous BFB cycles can lead to extensive chromosomal rearrangements including large terminal deletions or amplifications located within the chromosome (Lo et al., 2002; Roylance, 2002; Gisselsson, 2003; Storchova and Pellman, 2004; Shimizu et al., 2005; Bailey and Murnane, 2006; Ratis and Bapat, 2006).

Alternatively, anaphase bridges can remain attached during anaphase, presenting a physical barrier during cleavage and thus preventing successful cytokinesis (Fig. 1.9) (Gisselsson, 2003; Gisselsson and Höglund, 2005; Shimizu et al., 2005). Tetraploid derivatives may contain multiple centrosomes which, in turn, can cause multipolar cell division and consequent asymmetrical segregation of chromosomes, leading to unequal distribution of the DNA to the daughter cells (Mullins and Biesele, 1977; Gisselsson, 2003; Ratis and Bapat, 2006).

What is evident from the above is that whole chromosomal aberrations increase the frequency of acquiring further abnormalities, so that as tumours become more malignant, the frequency of both structural and numerical defects increases (Gisselsson, 2001; Maser and DePinho, 2002).

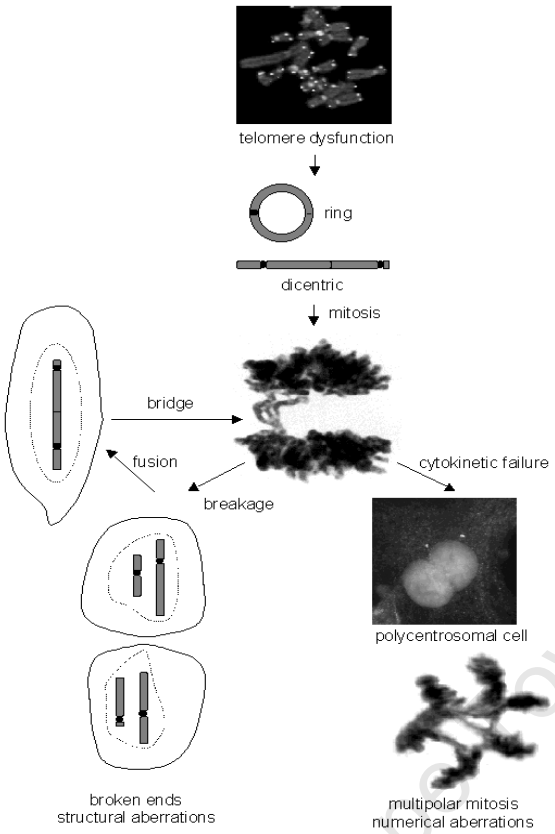

Figure 1.11. One possible mechanism by which CIN arises. Telomere shortening, in this instance due to the absence of detectable TTAGGG repeats (top microscopic image; fluorescence at the ends of some chromosome arms) may lead to chromosome fusions, and the formation of rings and dicentric chromosomes. These may form anaphase bridges, which can either break and initiate a series of BFB cycles, or prevent successful cytokinesis resulting in the generation of binucleate cells with multiple centrosomes. This may lead to the formation of a multipolar mitosis at the next cell division. Thus, telomeric dysfunction may result in both structural and numerical chromosome instability (from Gisselsson, 2001).

- ***Lagging chromosomes and micronuclei***

Lagging chromosomes can be defined as whole chromosomes or fragments thereof, which either fail to align properly at the metaphase plate or that lag behind the separating chromosomal masses during anaphase (Gisselsson, 2008). The misalignment of whole chromosomes has been shown to be a consequence of kinetochore proteins failing to attach to the mitotic spindle or due to a kinetochore becoming attached to both spindle poles (merotelic attachment). This prevents the timely movement of chromosomes toward opposite poles (Cimini et al., 2001; Biggins and Walczak, 2003; Gisselsson, 2008). Fragmented chromosomal material on the other hand, result following a double strand break in one chromosome arm, which generates a centric derivative and an acentric fragment (Gisselsson,

2001). The consequent acentric fragment, known as a double minute (DM) chromosome does not attach to the mitotic spindle during prometaphase and therefore appears as a lagging chromosome during metaphase and/or anaphase (Roylance, 2002; Gisselsson, 2008). Lagging chromosomes can result in the unequal distribution of the replicated DNA between daughter cells (Gisselsson, 2001; Roylance, 2002; Gisselsson, 2008). In addition, a nuclear envelope may form around lagging chromosomes at the end of mitosis leading to the formation of micronuclei (Gisselsson, 2008). Interestingly, a correlation between the formation of micronuclei and the presence of anaphase bridges has been demonstrated. Experiments utilising oral squamous cell carcinoma cells expressing GFP-tagged histones enabled researchers to monitor the outcome of anaphase bridges (Hoffelder et al., 2004). The authors showed that in at least 50% of the cases, these bridges fragmented during anaphase, appearing as lagging chromosomes which gave rise to micronuclei.

One of the many consequences of CIN is aneuploidy, which is the presence of an abnormal chromosome complement, both structurally and numerically, that is not a multiple of the haploid figure (Storchova and Pellman, 2004; Weaver and Cleveland, 2006; Storchova and Kuffer, 2008). Most stolid tumours are aneuploid and Theodore Boveri proposed, as early as the beginning of the 20th century, that aneuploidy plays a causative role in tumourigenesis (Boveri, 2008; Pihan and Doxsey 2003; Storchova and Pellman, 2004; Storchova and Kuffer, 2008). This proposal followed observations that tumours frequently underwent abnormal chromosome segregation during mitosis due to a multipolar spindle. Boveri therefore suggested that this may result in aneuploid cells and may be a direct cause of tumour development (Storchova and Pellman, 2004; Storchova and Kuffer, 2008).

The benefit of genomic instability to tumours has been illustrated by experiments that have shown that CIN may contribute to cancer cells developing cytotoxic drug resistance. This has been attributed to amplified regions harbouring extra copies of drug resistant genes (Gisselsson, 2008). For example, human cancer lines which display multidrug resistance frequently over-express the *multidrug resistance-associated protein* (*MRP*) gene, and this is typically due to chromosome amplification with additional copies of *MRP* genes located in homogenously staining regions and in DM chromosomes (Slovak et al., 1993; Deeley and Cole, 1997). In another example Li et al. (2005) investigated puromycin resistance in both breast and colon cancer cell lines and established that the degree of resistance was roughly proportional to the degree of aneuploidy in these cells. In addition to the above selected

examples, a multitude of studies are available which show that a compromised SAC or tetraploidy checkpoint results in cells that are more resistant to chemotherapeutic drugs (Wang et al., 2003a; Sudo et al., 2004; Cheung et al., 2005; Wang et al., 2007).

1.6 *Aims of this study*

Tbx2 can suppress senescence through a mechanism involving the repression of the cyclin dependent kinase inhibitors $p19^{ARF}$ and *p21* and TBX2 is over-expressed in melanoma, breast and pancreatic cancers. Although this strongly implicates TBX2 in the oncogenic process, whether its over-expression does indeed play a causative role in the development of cancer remains to be demonstrated. It was hypothesised, based on previous work in our laboratory, that the overexpression of TBX2 is instrumental in the development of cancer. This hypothesis was addressed by the following **aims**:

(i) to overexpress TBX2 in lung fibroblasts that express low levels of this protein and to establish if a transformed phenotype could be induced.

(ii) to knock down TBX2 in breast cancer and melanoma cell lines previously shown to overexpress TBX2 by a shRNA approach and to determine whether, at the very least, some features of the transformed phenotype could be reversed.

(iii) to determine the suitability of targeting TBX2 for chemotherapy by investigating the effect of overexpressing and silencing TBX2 on cellular response to cisplatin treatment.

Chapter 2 Materials and Methods

2.1 *Plasmids and DNA constructs*

All constructs used in this study were prepared according to standard techniques (Sambrook et al., 1989, pp 1.25-1.51). pcDNA 3.1(+) containing the full-length human TBX2 cDNA was supplied courtesy of Lingbeek et al. (2002).

2.1.1 Generation of pSuper.neo/GFP siRNA expression constructs

Sustained knockdown can be achieved through the use of small hairpin (sh)RNA, where an expression vector containing a polymerase III promoter can drive the synthesis of siRNAs. A summary of the cellular mechanism of gene silencing by RNAi will therefore be described (see fig. 2.1). Upon entry into a cell, double stranded RNA is recognised and processed into 21-23 nucleotide long siRNA molecules by Dicer, an RNase III enzyme (Bernstein et al., 2001). The siRNA molecules are next incorporated into the RNA-induced silencing complex (RISC) which is a multisubunit ribonucleoprotein complex with nuclease activity (Hammond et al., 2000). Unwinding of the bound siRNA duplex is an ATP-dependent process and leads to the activation of RISC (Nykanen et al., 2001). The anti-sense strand functions as a guide (Martinez et al., 2002) for targeting homologous mRNA molecules which become cleaved by RISC. The cleaved mRNA is degraded, resulting in loss of expression of the protein for which it encodes. To achieve sustained knockdown of a target mRNA, plasmids or viral vectors encoding short hairpin RNAs (shRNAs) can be stably transfected into the cell's genome, resulting in the expression of shRNAs that are exported to the cytoplasm where they enter the RNAi pathway (Brummelkamp et al., 2002b; reviewed by Shankar et al., 2005).

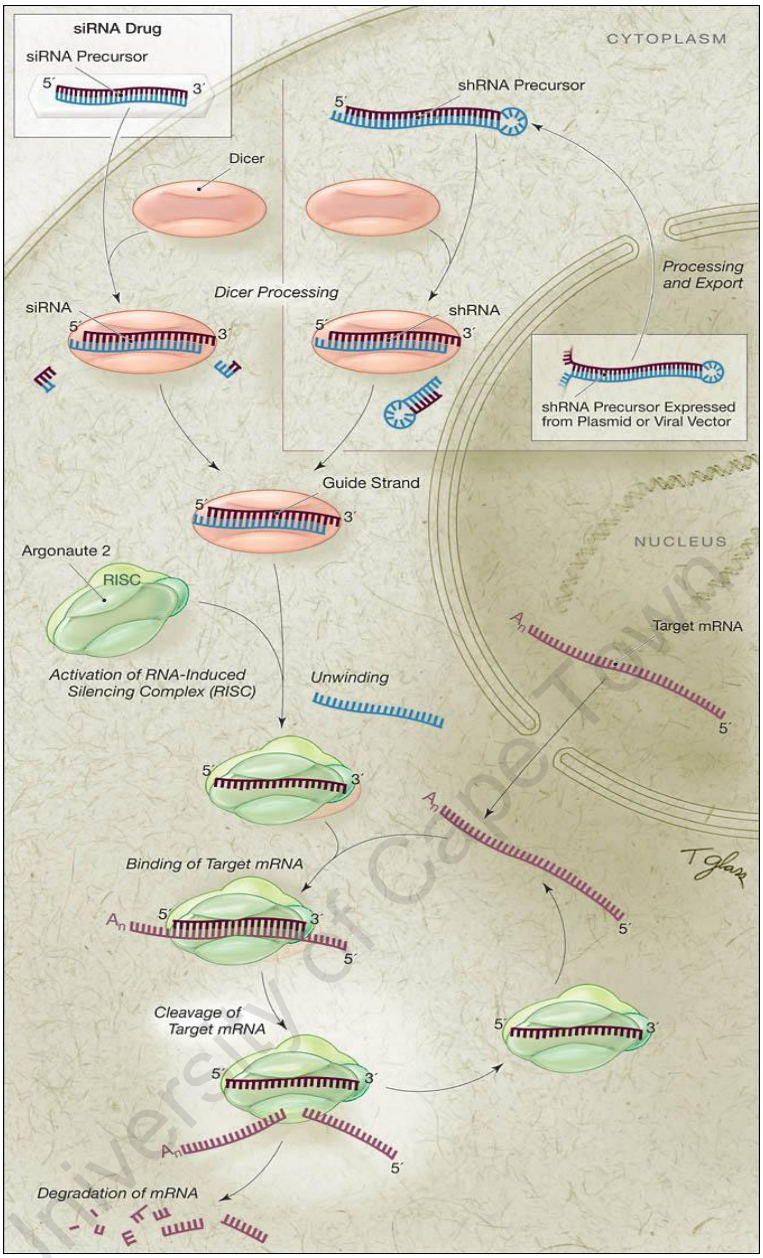

Figure 2.1. The cellular mechanism of gene silencing by RNA interference. Upon entry into the cell, long double stranded (ds) RNA is recognised by dicer and cleaved into short, 21-23 nucleotide long, siRNA molecules. The RNA induced silencing complex (RISC) binds the siRNA molecules which unwind, enabling the activation of RISC. The guide strand (5'-3') remains within the RISC complex and directs this complex to homologous mRNA sequences, which are cleaved in the center of the region of homology by Argonaute 2, an enzyme within the RISC complex. The cleaved mRNA is degraded by the cell, resulting in loss of expression of the protein for which it encodes. Alternately, plasmids or viral vectors encoding short hairpin RNAs (shRNAs) can be stably transfected into the cell's genome, resulting in the expression of shRNAs that are exported to the cytoplasm where they enter the RNAi pathway (from Shankar et al., 2005).

The pSuper.neo/GFP RNAi expression vector (oligoengine, Seattle, USA) was used to synthesise siRNA-like transcripts targeted towards silencing TBX2 expression (for vector map see appendix, section 6.7). Essentially, this DNA vector transcribes a small RNA transcript, lacking a polyadenosine tail, from the polymerase-III H1-RNA gene promoter. The sequence was designed so that, once transcribed, it will fold back on itself to form a stem-loop precursor, which is cleaved in the cell to form a functional siRNA transcript (See Fig. 2.2).

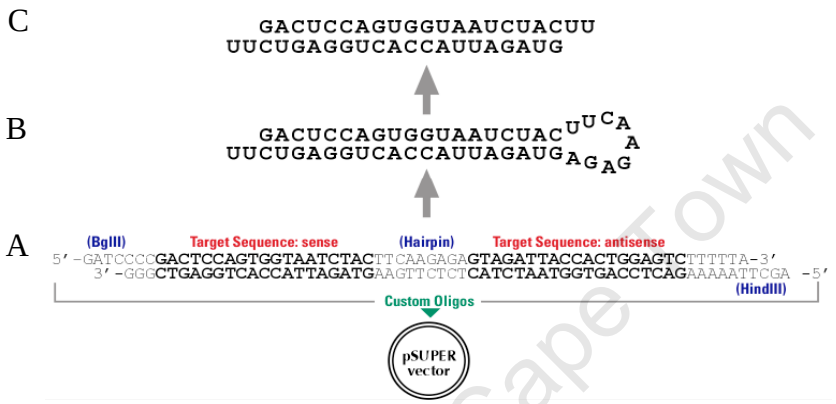

Fig. 2.2. Strategy to achieve stable knockdown of a target gene. The appropriate target sequence, separated by a 9bp spacer region (A), was cloned into pSuper.neo/GFP, a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts (oligoengine, Seattle, USA). Stable incorporation of this vector into the cells' genome enables transcription of a dsRNA oligonucleotide (B), which are processed in the cell to a functional siRNA (C) by the machinery of the RNAi pathway

Briefly, a 21 nucleotide sequence specifically targeted to TBX2 (ACAGCTGAAGATCGACAACAA) and a 21 nucleotide control, scrambled sequence (ATTTCTCCGAACGTGTCACGT) were synthesised (sequences courtesy of Qiagen, USA). Both the sense and anti-sense oligonucleotides were resuspended in Tris-EDTA, pH 8 to obtain a final concentration of 100 µM. To facilitate ligation, the sense and anti-sense oligonucleotides were incubated with T4 polynucleotide kinase (Promega, USA), an enzyme that catalyses the phosphorylation of the 5' hydroxyl group of oligonucleotides from an ATP donor. Kinase treatments were as follows: roughly 2 µg of either sense or anti-sense oligonucleotides was incubated with 10 units T4 polynucleotide kinase, 1 X kinase buffer and 10 mM ATP at 37°C for 30 minutes. The kinase was then inactivated at 65°C for 20 minutes. The sense and anti-sense strands were annealed by initially denaturing the strands at 95°C in annealing buffer (see appendix, section 6.1) and then cooling the oligonucleotides to room

temperature (RT) in a step-wise manner. The TBX2- and control- sequences were ligated into the pSuper.neo/GFP vector, which was used to transform *Escherichia coli* DH5α competent cells and correct transformants were selected following analyses by restriction enzyme digestion.

2.2 *Cell culture*

WI-38 human embryonic lung fibroblasts and their in vitro transformed counterparts WI-38 CT-1 cells (referred to as CT-1; Namba et al., 1980) were maintained in Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. The 501 human melanoma cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. The MCF7 breast adenocarcinoma cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 0.01 mg/ml insulin. Cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2, 65% humidity. Media was replaced every 2-3 days and cells routinely subjected to mycoplasma tests. Only mycoplasma free cells were used in experiments.

2.2.1 Mycoplasma test

Cells grown on a coverslip for a minimum of 24 h in antibiotic-free medium were fixed in a 1:3 mixture of glacial acetic acid and methanol for 5 sec, washed briefly with water to remove the fixing solution and then air-dried at RT for 5 min. Once dried, the DNA was stained with Hoechst 33258 (0.5 µg/ml) for 30 sec, washed briefly with water to remove excess stain and then mounted on a slide with mounting fluid (see Appendix, section 6.2). The cells were viewed immediately by fluorescence microscopy. Mycoplasma negative cells stained positive with Hoechst 33258 only in the nucleus, while cells infected with mycoplasma showed staining in both the nucleus and the cytoplasm.

2.2.2 Generation of stable cell lines over-expressing TBX2

The CT-1 cells were derived by cobalt 60 gamma-irradiation of the normal WI-38 lung fibroblast cell line (Namba et al., 1980). To generate stably transfected cell lines, CT-1 cells were transfected with either the empty expression vector pcDNA3.1 (+) or with this vector containing the full length human TBX2 cDNA (Lingbeek et al., 2002) using the standard

calcium-phosphate precipitation method (Teng et al., 2007). Among subcloned cell lines, cell lines chosen for subsequent analysis were: CT-TBX2(2) and CT-TBX2(3), containing the pcDNA3.1-TBX2 construct while CT-E and CT-E(2) contained the pcDNA3.1 empty vector. Individual clones were selected with 400 µg/ml G-418 antibiotic 48 h post transfection.

2.2.3 Generation of stable cell lines in which TBX2 was knocked down

The MCF-7 breast cancer and 501 melanoma cells have been shown to over-express TBX2 and were thus selected to generate stable cell lines knocking down TBX2 (Jacobs et al., 2000; Vance et al., 2005). Cells were stably transfected with the pSuper.neo/GFP vector, containing either a TBX2- or a scrambled control- sequence, using the FuGENE HD Transfection reagent (Roche, Germany), according to the manufacturers' instructions. Briefly, MCF7 or 501 cells were plated in 35mm tissue culture dishes so as to be 50-70% confluent on the day of transfection. Three microlitres of the transfection reagent diluted into 97 µl serum-free media (without antibiotic) was prepared at RT and allowed to stand for 5 min. The diluted transfection reagent was added in a dropwise fashion to the DNA (a 3:1 ratio of µl FuGENE HD: µg DNA), mixed and incubated at RT for 15 min. The transfection reagent:DNA complex was added dropwise to the cells. Stable transfectants were selected for with G-418 antibiotic (Promega, USA) (as described below) 48 h post transfection and individual clones selected. Among subcloned cell lines, those chosen for subsequent analysis were: MCF-7 shTBX2(3) and MCF-7 shTBX2(5) and 501 shTBX2(8) and 501 shTBX2(10) expressing the shTBX2 construct while MCF-7 shcontrol(5) and MCF-7 shcontrol(8) and 501 shcontrol(3) and 501 shcontrol(6) expressed the shcontrol construct.

To ensure that the stable cell lines were continuously expressing the desired plasmid, cells were treated with the appropriate antibiotic. G-418, a neomycin analogue, was used for the pSuper.neo/GFP vector. To determine the effective concentration of antibiotic for each cell line, untransfected cells were seeded in a 6-well plate so that cells were approximately 60% confluent on the first day of treatment. Cells were treated with concentrations of G-418, ranging from 0 – 800 µg/ml and monitored daily. The lowest concentration which resulted in complete cell death after approximately 10 days was selected. For both the MCF-7 and 501 cells 400 µg/ml G-418 was chosen.

2.2.4 Treatments

2.2.4.1 Cell cycle synchronization

Cells were synchronised in specific phases of the cell cycle by a double thymidine block (15 h 2 mM thymidine (Sigma, USA), 9 h release, 15 hr thymidine) to obtain a population of cells at the G1/S boundary, or were released into fresh medium for 2-4 h to obtain S-phase cells. Cells were arrested in the G2/M-phases with a single thymidine block followed by 16 h with 0.1 μg/ml nocodazole (Sigma, USA). The non-adherent mitotic cells were isolated from the medium following gentle shaking, while the adherent cells were collected as the G2 population. Successful cell cycle blocks were confirmed by flow cytometry using a Beckman Coulter Cytomics FC500 or FACSCalibur flow cytometer, as described in section 2.7.

2.2.4.2 Cisplatin treatments

To determine drug resistance, cell viability was monitored following incubation in varying doses of cisplatin (Pfizer, South Africa). Cells were seeded in quadruplicate in a 96-well plate (3000 cells/well) and incubated for 72 h with cisplatin at concentrations ranging from 0 to 166.7 μM in culture medium. Cell viability was determined using the MTT assay according to the manufacturer's instructions (Roche, Germany). As an assay for proliferation, cells were plated on glass coverslips in 35 mm tissue culture dishes at a density of 3 X 10^4 cells/ml and allowed to attach. The cells were then grown in medium containing cisplatin at concentrations ranging from 0 to 50 μM in the presence of 10 μM BrdU for 48 h, and processed as described in section 2.3.3.

In order to induce DNA damage, cells were treated with 5 or 10 μM cisplatin for the time course indicated. In all cases untreated cells were incubated with 150 mM NaCl, because it was the vehicle in which cisplatin was dissolved.

2.3 *Growth curves*

Three methods were utilised to determine short term cell growth, namely: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Kit (Roche, Germany), counting cells on a haemocytometer and the 5-bromo-2-deoxyuridine (BrdU) incorporation assay, which will be described below.

2.3.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To determine cell growth using the MTT Cell Proliferation Kit, cells were seeded in quadruplicate in a 96-well plate (1000 cells/well) and cell viability determined according to the manufacturer's instructions. Briefly, 24 h prior to harvesting 10 µl of pre-warmed MTT labelling reagent was added to each well and 4 h later 100 µl of pre-warmed solubilisation solution was added to the cells. The spectrophotometrical absorbance of the samples was determined at a wavelength of 595 nm using a 96-well plate reader, with the absorbance of the medium only control being subtracted from the samples. Cell proliferation was determined over 9 days.

2.3.2 Counting using a haemocytometer

Cell counts, using a haemocytometer, were determined as described previously (Prince et al., 2003). Cells were seeded in triplicate in a 12-well plate at a density of 5 X 10^3 cells/well for the 501 melanoma cells and 1 X 10^4 cells/well for the CT-1 and MCF-7 cell lines. Cells were collected by trypsinization and counted on a haemocytometer at 2-3 day intervals.

2.3.3 5-bromo-2-deoxyuridine (BrdU) incorporation assay

Cells were seeded on glass coverslips in 35 mm tissue culture dishes at a density of 4 X 10^4 cells/ml and allowed to attach. The cells were then incubated in medium containing 10 µM BrdU for 1 h and 8 h followed by fixing with Carnoy's Fixative (see appendix, section 6.3) at -20ºC for 20 min. For immunostaining, the cultures were incubated in 2 N hydrochloric acid at 37ºC for 1 h, neutralised in 0.1 M borate buffer (see appendix, section 6.3), rinsed with PBS (see appendix, section, 6.5) containing 0.05% Tween-20 (PBS/T) and incubated in PBS/T with 5% swine serum for 30 min at 37ºC. BrdU was detected with the anti-BrdU mouse monoclonal antibody (6 µg/ml, Roche, Germany) for 30 min at 37ºC, followed by a secondary IgG coupled to Alexa 488 (1:1000, Molecular Probes, USA) for 30 min at 37ºC. Cells were rinsed with PBS/T, incubated in propidium iodide (20 µg/ml, Sigma, USA) for 20 min, mounted onto slides and visualised by fluorescence microscopy.

2.4 *Microscopy*

Cells grown on glass coverslips were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (see appendix, section 6.4) for 20 min at RT. Cells were then permeabilised in 0.2% Triton X-100 in PBS for 10 min at RT. The cells were viewed at this stage for fixed cell phase contrast images. For immunofluorescence, cells were blocked for 1 h with blocking buffer (5% swine serum in PBS) at RT and incubated with the appropriate primary antibody diluted in blocking buffer at 4°C O/N in a humidifying chamber. Cells were washed in PBS and incubated with the appropriate fluor-conjugated secondary antibodies (1:1000) for 2 h at RT in the dark. Cells were again washed in PBS and the DNA was stained by incubating the cells with 1 μg/ml DAPI (4',6-diamidino-2-phenylindole) (Sigma, USA) or 20 μg/ml propidium iodide (Sigma, USA) diluted in PBS for 10 min at RT in the dark. Cells were washed, the coverslips mounted onto glass slides with Movial mounting medium (Hoechst, Germany) containing n-Propyl gallate (Sigma, USA) to prevent the signal fading and the cells visualised by fluorescence microscopy using an Axiovert fluorescent microscope (Zeiss, Germany). Primary antibodies were: mouse monoclonal anti-Tbx2 62-2 (1:750) and rabbit polyclonal anti-phospho-Histone H3 (ser10) (1:200) (Upstate biotechnology, Charlottesville, USA) and anti-phospho-H2A.X (ser139) (γ-H2AX) (1:500) (Cell Signaling, Technology Inc., Beverly, MA). Secondary antibodies were: alexa 488 goat anti-mouse (Molecular probes, Eugene, USA) and Cy3 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., USA). Negative controls were as for above, but the primary antibody was excluded.

2.5 *Western blot analysis*

Cells were harvested on ice by scraping with a 1 ml plunger in RIPA buffer (see appendix, section 6.5). Whole cell extracts were incubated on ice for a minimum of 30 min, centrifuged at 12,000g for 20 min at 4°C and the supernatants recovered. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA), according to the manufacturer's instructions with bovine serum albumin as the standard. Equal amounts of protein were loaded and separated on 6-15% SDS-polyacrylamide gels (see appendix, section 6.5) and transferred to Hybond ECL nitrocellulose membrane (Amersham, Biosciences, USA). Membranes were blocked for 1 h at RT with PBS containing 5% non-fat dry milk and

probed with appropriate primary antibodies O/N at 4°C with shaking. Membranes were washed in PBS/0.1% TWEEN (PBS/T) and incubated with peroxidase-conjugated anti-mouse or anti-rabbit (BioRad, Hercules, CA, USA) or anti-goat antibody (1:5000) (Santa Cruz Biotechnology, CA, USA). Membranes were again washed in PBS/T and visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The primary antibodies and appropriate dilutions were: mouse monoclonal anti-Tbx2 62-2 (1:2000) (Prince et al., 2004) or goat polyclonal anti-Tbx2 (1:1000) (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-p38 (1:5000) (Sigma, Missouri, USA) (Abrahams et al., 2008), mouse monoclonal anti-cyclin B1 (1:1000) (Transduction Laboratories) (Davis et al., 2008), rabbit polyclonal anti-phospho-Histone H3 (ser10) (1:1000) (Upstate Biotechnology) (Teng et al., 2009), rabbit polyclonal anti-phospho-H2A.X (ser139) (γ-H2AX) (1:1000) (Wellen et al., 2009), anti-phospho p38 (1:1000) (Abrahams et al., 2008), anti-phospho p44/p42 (Erk 1/2)(1:1000) and mouse monoclonal anti-phospho JNK (1:1000) (Cell Signaling, Technology Inc., Beverly, MA) (Teng et al., 2009) and mouse monoclonal anti-tubulin (1:500) (Davis et al., 2008), anti-ATM (1:500) (Feng et al., 2007), goat polyclonal anti-ATR (1:500) (Wang et al., 2003b), rabbit polyclonal anti-p53 (1:1000) (Feng et al., 2007), anti-cyclin A (1:1000) (Vaňhara et al., 2007), anti-p21 (1:1000) (Davis et al., 2008), anti-PARP-1/2 (1:1000) (Rieber and Rieber, 2003) and anti-$p19^{ARF}$ (1:200) (Chaturvedi et al., 2003) (Santa Cruz Biotechnology, CA, USA).

To analyse the membrane with a primary antibody targeted towards a different protein, bound primary antibody was removed as follows: membranes were rinsed with PBS/T, stripped in stripping buffer (see appendix, section 6.5) for 30 min at 50°C and washed 2X PBS/T.

2.6 *Flow cytometry*

Cells were collected by trypsinisation, washed twice with PBS, resuspended in 2 ml of cold PBS and counted on a haemocytometer to determine the volume of propidium iodide solution that will be added. Cells were fixed in 8 ml of 70% cold ethanol for at least 30 min at -20°C. Fixed cells were collected by centrifugation at 1500 rpm for 5 min at RT, washed twice with PBS and centrifuged at 6000 rpm for 1 min at RT. Before flow cytometry analyses, the samples were treated with RNase A (50 μg/ml) for 15 min at 37°C and immediately stained for 30min at RT with propidium iodide solution (see appendix, section 6.6), to yield a final

concentration of 1 X 10^6 cells/ml. A minimum of 50 000 cells/sample were subjected to analysis using either a Beckman Coulter Cytomics FC500 or FACSCalibur flow cytometer.

2.7 *Metaphase spreads*

Cells grown to 70% confluency on glass cover slips were arrested in metaphase by treatment with 0.1 µg/ml of colcemid for 1 h at 37°C. Following hypotonic treatment in 0.56% KCl solution at 37°C for 1 h, the cells were fixed in 1:3 acetic acid:methanol fixative at 37°C for 1 h. Evaluation of the chromosome sets was carried out after conventional Giemsa (EM Science, Gardena, CA) staining. Metaphase chromosome spreads were prepared on acid-cleaned microscope slides using the standard method (Priest, 1977). For G-banding staining, slides were incubated 3 seconds to 1 minute in a trypsin (DIFCO 1:250) solution (0.1 g Trypsin in 100 ml isotonic buffer). Slides were rinsed for a few seconds in a jar with FCS (2-3 ml FCS in 50 ml isotonic buffer) and then rinsed in isotonic buffer and incubated for 1.5-5 minutes in a Coplin jar with Giemsa stain (EM Science, Gardena, CA).

2.8 *Transformation assays*

2.8.1 Growth factor dependence assay

For the growth factor-dependence assay, cell proliferation was compared in medium containing either 2% or 10% FBS, using short-term growth assays as described in section 2.3.2 above.

2.8.2 Anchorage independence assays

- ***Soft agar assay***

For soft agar assays, 35 mm tissue culture dishes were coated with a 1% agar/medium layer to prevent cells from attaching to the bottom of the dish. Dishes were incubated at 4 °C O/N to allow the agar to solidify. Cells were resuspended in 0.35% agar/medium slurry and plated on top of the 1% agar/medium layer at a concentration of 5000 cells per 35mm dish. In order to prevent the agar/medium slurry from desiccation, 1 ml of fresh medium was added per dish. The dishes were incubated at 37 °C in the presence of 5% CO_2 for 2-3 weeks and a few drops of fresh medium was added twice a week. Cell viability was determined by O/N incubation with p-iodonitrotetrazolium-chloride (Sigma, USA), which forms a purple formazan dye when reduced. Pictures were taken using a non-phase contrast lens (Sony cyber

shot, DSC-T20). Higher magnification images were taken using an Axiovert fluorescent microscope (Zeiss, Germany) (10x magnification).

- ***Methylcellulose assay***

The wells of a 96-well plate were coated with 12 mg/ml poly (2-hydroxyethyl methacrylate) (polyHema) (Sigma, USA) in order to prevent cells from adhering to the bottom of the well. Cells were seeded at a concentration of 2 X 10^3 and 1 X 10^3 cells/well for the MCF-7 and 501 cell lines respectively in the appropriate medium (described above) containing 0.8% methylcellulose (Sigma, USA). Cell viability was monitored for 7-10 days by the addition of p-iodonitrotetrazolium-chloride and images were obtained using an Axiovert fluorescent microscope (Zeiss, Germany).

2.8.3 Cell migration assay

Cell migration in culture was measured using a two-dimensional in vitro scratch motility assay. Cells were grown to confluence in 35 mm tissue culture dishes. A linear wound was made by scratching through the monolayer using a sterile 200 µl pipette tip. To remove cell debris, the growth medium was replaced, and to prevent cell proliferation Mitomycin C (Sigma, USA) was added at a final concentration of 10 µg/ml. Several markings were made along the edges of the scratch line which were used as reference points and the wound widths measured at the time of the scratching (0 h) and thereafter at 1 – 2 h intervals. To induce migration in the MCF-7 cells, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) was added at a final concentration of 10 nM. TPA induces migration in poorly migrating cell lines, including MCF-7 cells, by downregulating protein kinase Cδ, a factor which suppresses cell migration (Jackson et al., 2005). Pictures were taken using a phase contrast microscope and migration distances were measured using Axio software (Zeiss, Germany). The difference in width represents the distance migrated in µm.

2.8.4 Tumour forming ability in nude mice

In vivo tumour formation was assayed by subcutaneously injecting cultured cells into the flanks of 6-8 week-old MF-1 nude mice. For the MCF-7 cell lines 1 X 10^6 cells, resuspended in 50 µl sterile PBS, were injected into female mice. For the 501 cell lines 2 X 10^6 cells were resuspended in 100 µl sterile PBS and injected into both male and female mice, the sexes being approximately equally distributed between shTBX2 and shcontrol cell lines. Seven

mice in total, maintained in pathogen-free environments, were used per cell line and were monitored weekly for tumour formation. Lymph node size was determined in 2-dimension using vernier callipers and the mass of the lymph nodes measured on a scale. Tissue specimens for microscopy were collected in 10% formal-saline and further processed in an automatic tissue processor (Shandon Duplex) for routine paraffin wax embedding. Tissue sections were cut, mounted onto clean glass slides, stained with haematoxylin and eosin and viewed using a light microscope (Zeiss, Germany).

Chapter 3 Results

In addition to their critical roles in embryonic development, T-box factors have also been implicated in cell cycle regulation and in the genesis of cancer. Both Tbx2 and Tbx3, for example, can prevent senescence in mouse embryonic fibroblasts and ST.Hdh^{Q111} striatal cells through a mechanism involving their ability to repress the negative cell cycle regulators *p19^{ARF}* (Jacobs et al., 2000; Carlson et al., 2001; Brummelkamp et al., 2002a) and *p21* (Prince et al., 2004). Ectopic expression of Tbx3 together with oncogenic Ras or Myc in embryonic mouse fibroblasts leads to cellular transformation and suppression of apoptosis (Carlson et al., 2002). Furthermore, both TBX2 and TBX3 are expressed in the developing breast (Jerome-Majewska et al., 2005) and their genes are amplified and/or over-expressed in some breast tumours (Packham and Brook, 2003; Sinclair et al., 2002) and in certain breast cancer cell lines (Fan et al., 2004). TBX2 is also over-expressed in 50% of pancreatic cancer cell lines (Mahlamaki et al., 2002) and in melanomas where it was shown to function as an anti-senescence factor (Vance et al., 2005). While these results suggest that increased levels of T-box factors may contribute directly to oncogenesis this has not been unequivocally shown. Furthermore, whether their involvement in cancer is exclusively through suppressing senescence or whether additional mechanisms are involved remains to be elucidated. This study addressed these two questions by establishing cell culture models in which TBX2 was either (1) over-expressed in cells with low levels of TBX2 or (2) knocked down in cancer cell lines that express high levels of this protein. In both instances the effects of altering TBX2 protein levels on key features of tumourigenesis were examined.

3.1 *Over-expression of TBX2 in transformed human lung fibroblasts*

Although TBX2 has been reported to be up-regulated in a variety of cancers, our laboratory has previously shown that while TBX2 is expressed in the normal WI-38 lung fibroblast cell line, it is down-regulated in several of its transformed counterparts, including the Co-60 gamma irradiated CT-1 cell line (Teng et al., 2007). In order to investigate the implication of this down-regulation of TBX2 upon cellular transformation, an expression construct in which the CMV promoter drives expression of the human TBX2 was stably transfected into CT-1 cells by a previous PhD student in our laboratory (Teng et al., 2007). A number of G418-

resistant clones, indicating successful expression of the recombinant construct, were isolated and further analysed in this study, as described below.

3.1.1 Re-expression of TBX2 in CT-1 cells resulted in a decrease in cell proliferation rate and an altered morphology

To identify TBX2 over-expressing clones that could be further analysed, a number of G418 resistant clones were screened for TBX2 levels by western blot analyses. Figure 3.1(a) shows the presence of TBX2 protein in two representative clones (CT-TBX2(2) and CT-TBX2(3)) which was absent in cells transfected with the empty vector (CT-E) and the parental CT-1 cells. Figure 3.1(b) shows that WI-38 cells had much higher levels of TBX2 protein compared to the CT-TBX2 clones and therefore any subsequent changes observed in the CT-TBX2 cells were not due to abnormally high levels of TBX2.

While expanding the CT-E and CT-TBX2 cell lines, it was observed that the TBX2-expressing cells grew much more slowly than the CT-E control cells. To explore this further, the growth rate of the CT-E control cells was compared to that of the CT-TBX2 cells using the MTT cell proliferation assay. However, contrary to what was observed during cloning expansion, the results indicated that the CT-TBX2 cells were proliferating faster than the control cells (Fig. 3.2a). The rate of cell proliferation was therefore re-measured using a haemocytometer to count cells over a 9-day culture period. In keeping with the initial observations, the CT-TBX2 cells had a decreased cell proliferation rate (Fig. 3.2b), growing approximately 2.5 times more slowly than the CT-E control cells on day 9. To clarify the effect of TBX2 on cell proliferation, BrdU incorporation assays were performed in which CT-E and the two CT-TBX2 cell lines were pulsed with BrdU for 1 and 8 hours. BrdU is a synthetic thymidine analogue and thus only incorporated by actively proliferating cells. Figure 3.2(c) shows the total number of BrdU positive cells expressed as a percentage of the total number of cells from 10 fields of view for each cell line. Whereas similar numbers of CT-E cells had incorporated BrdU as the CT-TBX2 cell lines at 1 h, after 8 h 97% of the CT-E cells had incorporated BrdU compared to 66% and 44% for the CT-TBX2(2) and CT-TBX2(3) cell lines respectively. These results thus confirmed that TBX2 does indeed have a negative effect on cell proliferation of transformed fibroblasts.

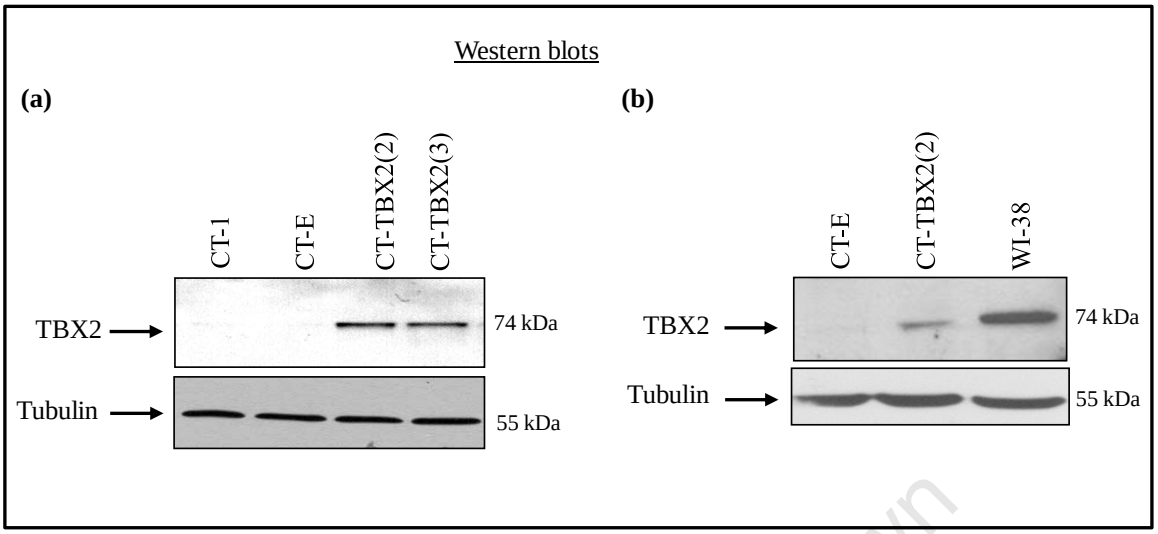

Figure 3.1. Establishment of CT-E and CT-TBX2 cell lines. (a) Over-expression of TBX2 in two CT-TBX2 cell lines compared to undetectable levels in the CT-1 and CT-E control cells. Protein extracts (40 µg) harvested from the indicated cell lines were analysed by SDS-PAGE (8%) and western blotting using an anti-TBX2 antibody. Tubulin was used as a loading control. (b) TBX2 protein levels in the CT-TBX2 clones were much lower than the endogenous TBX2 in WI-38 normal lung fibroblasts. Protein extracts (40 µg) were harvested from the indicated cell lines and analysed as for (a).

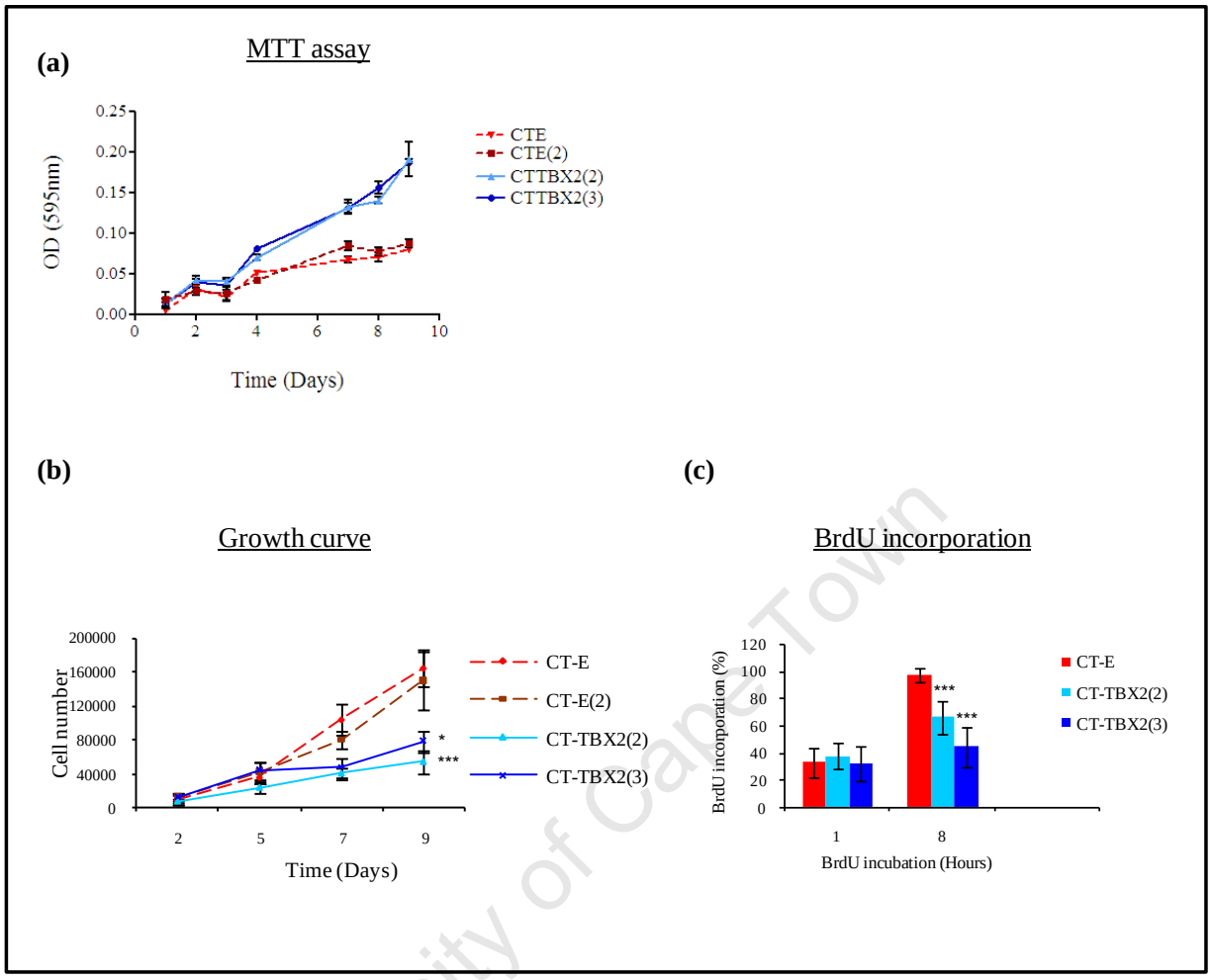

Figure 3.2. TBX2 over-expression reduces cell proliferation. (a) The MTT cell proliferation assay shows that the CT-TBX2 cells proliferate at a faster rate compared to the CT-E cells. Cells were seeded in quadruplicate at a density of $1X10^3$ cells per well of a 96-well plate. Growth curve assays were performed over a 9-day period and proliferation determined using the MTT cell proliferation assay. (b) and (c) Cell counts and BrdU incorporation assays show that the CT-TBX2 cells proliferate more slowly as compared to the CT-E cells. (b) Cells were seeded in triplicate at a density of $1X10^4$ cells per well of a 12-well plate. Growth curve assays were performed over a 9-day period and cells harvested by trypsinisation and counted on a haemocytometer. The number of cells for both CT-TBX2 cell lines were statistically significantly less as compared to the CT-E cell lines on day 7 and 9 (Student's unpaired t test, two-tailed, $p < 0.02$). Results represent the means ±s.d. of two individual experiments. (c) Cells were seeded on sterile coverslips, pulsed with BrdU for 1 and 8 h and harvested for immunocytochemistry using an anti-BrdU antibody. BrdU-positive nuclei were visualised by fluorescence microscopy. Results show an average of 10 fields of view with BrdU-positive cells being expressed as a percentage of total cells counted. The percentage BrdU incorporation for both CT-TBX2 cell lines were statistically significantly less as compared to the CT-E cell line on day 8 (Student's unpaired t test, two-tailed, $p < 0.0002$).

It is important to note that the MTT assay measures mitochondrial activity and is therefore not an accurate method of measuring proliferation between different cell lines. The results from the MTT assay did, however, suggest that the CT-TBX2 cells had more mitochondria per cell, possibly due to a larger cell volume. This theory was supported by observations that the CT-TBX2 cells had a morphology which was significantly different to that of the CT-E and untransfected cells. The CT-TBX2 cells were larger and their cell borders less defined (Fig. 3.3a).

3.1.2 TBX2 over-expression induced a G2/M arrest

Several of the CT-TBX2 cells had large nuclei or were binucleate suggesting that they fail to undergo cytokinesis. This was confirmed by flow cytometry which revealed that TBX2-expressing cells had a cell cycle profile very different to normal cycling cells, such as that seen for the CT-E cells (Fig. 3.3b). Whereas only 15% of the CT-E cells were 4N, 73% of the CT-TBX2 cells had a DNA content of 4N or greater. These results suggested that the TBX2-expressing cells were arrested at either a G2 or M checkpoint. The possibility that TBX2 may be exerting an effect on key cell cycle proteins involved in regulating these two checkpoints was explored by comparing the levels of p53, p21, cyclin B1 and $p14^{ARF}$ (human homologue of $p19^{ARF}$) in CT-E and CT-TBX2 cells. No detectable differences in the levels of the p53, p21 and cyclin B1 proteins were seen when they were standardised to tubulin levels in western blot analyses (Fig. 3.3c). Interestingly, $p14^{ARF}$ levels were higher in the CT-TBX2 cells which could be as a result of the cells being blocked in G2. Indeed, $p14^{ARF}$ has been shown to be able to induce a G2 arrest, in both a p53-dependent (Stott et al., 1998) and -independent manner (Normand et al., 2005). Furthermore, as shown in figure 3.3(b) (see arrow), TBX2 induced apoptosis in a small number of CT-TBX2 cells as indicated by a sub-G1 peak, indicating that over-expression of TBX2 resulted in a certain level of stress on these cells.

It is important to note here that the effects of over-expressing TBX2 in the CT-1 cells could be reproduced in another cell type in our collaborators laboratory (Davis et al., 2008). The K1735 murine melanoma cell line, that does not express endogenous Tbx2, was infected with a pBabePuro retrovirus expressing SV5-tagged Tbx2. Compared to the parental cells, several Tbx2-expressing clones proliferated at a reduced rate and exhibited an altered morphology.

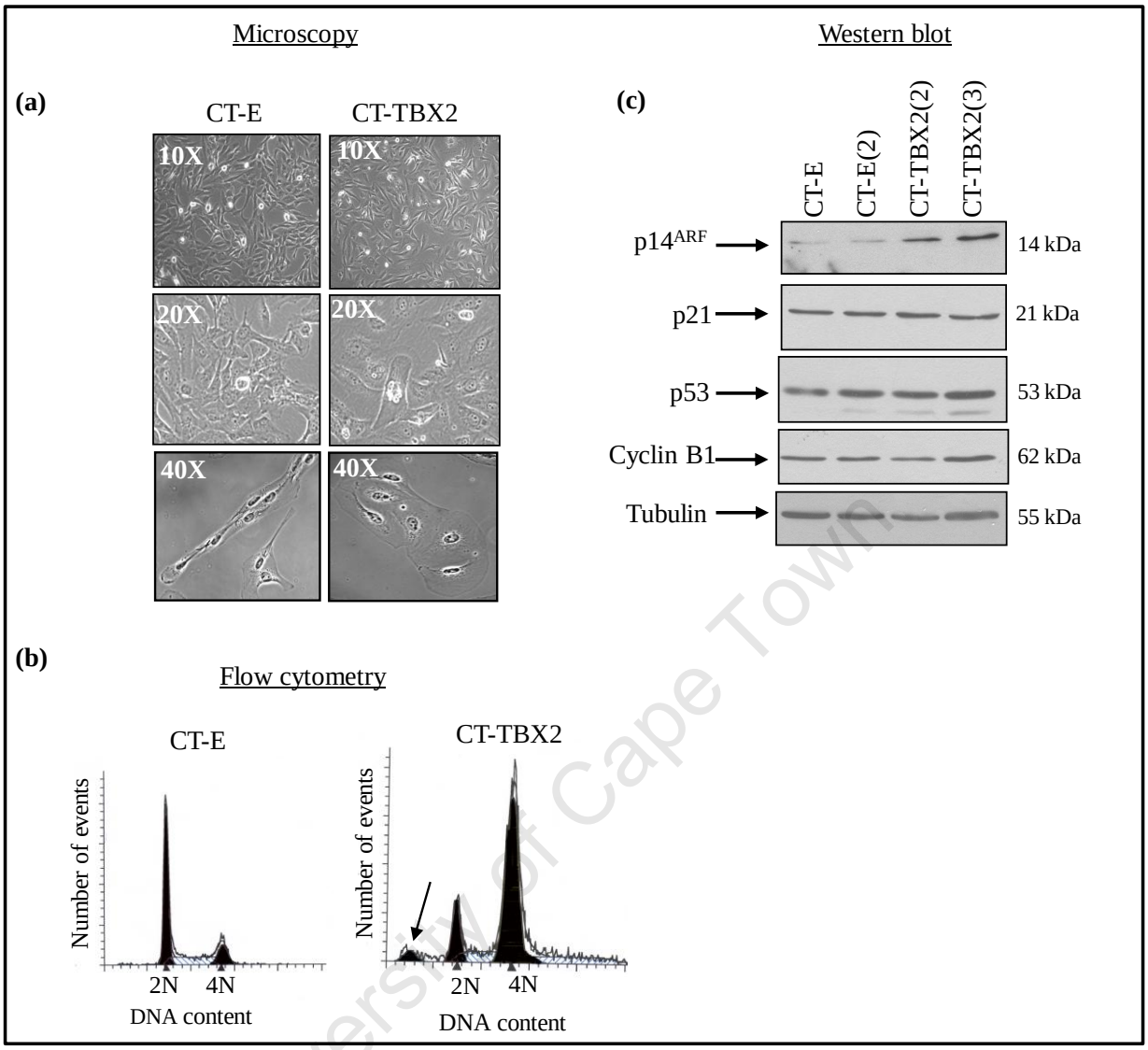

Figure 3.3. TBX2 over-expression affects cell morphology and induces a G2/M cell cycle arrest. (a) CT-TBX2 cells were larger and flatter than CT-E cells. Phase contrast images of CT-E (left panel) and CT-TBX2 (right panel) cells photographed at indicated magnifications. (b) CT-TBX2 cells were initially blocked in G2/M (4N). The cell cycle distribution of CT-TBX2 and CT-E cells was determined by staining cells with propidium iodide and measuring their DNA content using flow cytometry. The arrow points to CT-TBX2 cells that show a sub-G1 peak characteristic of cells undergoing apoptosis. (c) TBX2 expression does not affect the levels of key cell cycle regulators, but results in increased $p14^{ARF}$ levels. Protein extracts (30 µg) harvested from the indicated cell lines were analysed on 10-15% SDS-PAGE and by western blotting using antibodies to indicated proteins. Tubulin was used as a loading control.

Furthermore, flow cytometry analysis revealed that the Tbx2-expressing clones displayed an abnormal cell cycle profile with a substantial increase in the G2 fraction and, in some instances, the emergence of an 8N (G2/M tetraploid) peak was also seen. Taken together these results confirmed that over-expression of Tbx2/TBX2 leads to defects in mitosis and that the effects of TBX2 in the CT-TBX2 cells were not species or cell type dependent.

3.1.3 TBX2 over-expression induced mitotic defects and polyploidy

With increasing passage of the CT-TBX2 cell lines, flow cytometry revealed the gradual appearance of an 8N (G2/M tetraploid) peak (Fig. 3.4a). These results are in agreement with previous reports that spindle assembly checkpoint (SAC) activation is often only transient, with some cells "slipping" past the arrest and producing a tetraploid population due to defective cell division (Andreassen et al., 1996; Minn et al., 1996; reviewed by Storchova and Pellman, 2004). These cells should ordinarily arrest in G1 at the tetraploidy checkpoint and the relevance of this result is addressed later in 3.1.4.

The next experiment investigated whether the CT-TBX2 cells do indeed have double the number of chromosomes compared to the CT-E cells. Metaphase spreads confirmed that all CT-TBX2 cells analysed had at least 160 chromosomes compared to a maximum of 85 chromosomes seen in CT-E cells (Fig. 3.4b). As discussed earlier, it is not unusual for transformed cells to have an increased DNA content, therefore it was not surprising that this was the case for the CT-E cells. However to confirm that this result was not due to experimental error the CT-1 and WI-38 cells were also analysed by metaphase spreads. The chromosome numbers counted for the CT-1 cells confirmed that obtained for the CT-E cells (data not shown) and, as expected, the normal WI-38 cells had 46 chromosomes (Fig. 3.4b).

The observation that the CT-TBX2 cells had an increased number of chromosomes suggested that over-expression of TBX2 may be enhancing chromosomal instability (CIN), as this is frequently associated with errors in cell division that lead to polyploidy. Indeed, Giemsa-banding analysis on metaphase chromosomes revealed that, compared to CT-E cells, CT-TBX2 cells had an increased frequency of several chromosomal abnormalities indicative of CIN (Fig. 3.4c). For example, whereas dicentric and giant marker chromosomes were never seen in CT-E control cells, 78% of the CT-TBX2 metaphase spreads had between one and

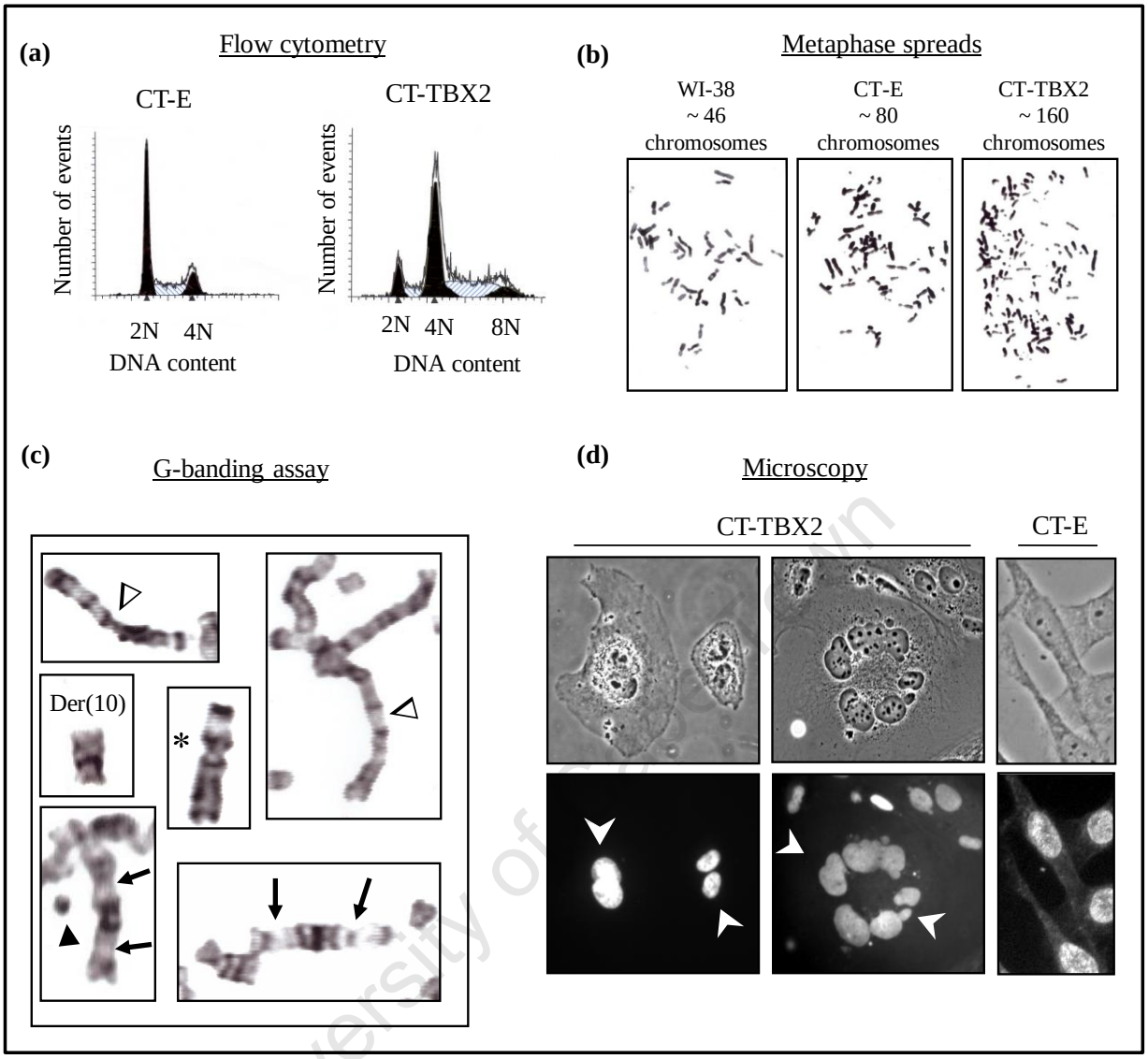

Figure 3.4. TBX2 over-expression results in cells exhibiting several features of polyploidy and chromosomal instability (CIN). (a) With increasing passage, CT-TBX2 cells undergo mitotic slippage resulting in the emergence of a G2/M tetraploid (8N) population. The cell cycle distribution of CT-TBX2 and CT-E cells was determined by staining cells with propidium iodide and measuring their DNA content using flow cytometry. (b) CT-TBX2 cells have double the DNA content of CT-E cells. WI-38, CT-E, and CT-TBX2 cells were plated on sterile coverslips, arrested in metaphase with colcemid and the DNA stained with Giemsa. (c) CT-TBX2 cells have enhanced CIN. Giemsa-banding analysis for CT-TBX2 cells reveal dicentric chromosomes (arrows), giant marker chromosomes (Δ), very small marker chromosomes (▲), unidentifiable or marker chromosomes (*) and a derivative of chromosome 10 (Der(10)). (d) CT-TBX2 cells display genomic instability. Phase-contrast images (upper panel) of CT-TBX2 cells at 40X magnification with corresponding immunofluorescence images of DAPI labeled nuclei (lower panel). Arrowheads indicate examples of a lobular nucleus, a binucleate cell and a multinucleate cell with micronuclei.

three dicentric chromosomes (arrows) and 44% displayed giant marker chromosomes (Δ). In addition, while metaphase spreads of both the CT-E and CT-TBX2 cells contained very small marker chromosomes, they occurred with twice the frequency in the CT-TBX2 cells (►). urthermore, compared to the CT-E control cells, CT-TBX2 cells had almost no apparently normal chromosomes. Numerous derivative and unidentifiable chromosomes (asterisk), which must have resulted from translocations or chromosomal fusions, predominated. CT-TBX2 cells also displayed nuclear abnormalities characteristic of CIN. For example, the presence of micronuclei was detected in 23% of cells analysed and many cells were binucleate or had large lobular nuclei (Fig. 3.4d), with the average size of CT-TBX2 nuclei being 0.17 μm² compared with an average of 0.11 μm² obtained for the CT-E cells (data not shown).

3.1.4 TBX2 over-expression resulted in abnormalities in anaphase

In order to determine how over-expression of TBX2 increases the ploidy of the CT-1 cells, it is necessary to review the mechanisms by which this state can occur. Tetraploid cells can result due to errors in multiple processes in cell division. However several cell cycle checkpoints exist to detect these errors. For example, cells in metaphase that cannot satisfy the SAC should arrest at this stage (Rieder et al., 1994; 1995; reviewed by Kops, 2008). However, if these defects do not trigger mitotic catastrophe, prolonged activation of the SAC results in cells bypassing this checkpoint and being arrested at the G1 tetraploidy checkpoint (Minn et al., 1996; Margolis et al., 2003). Cells which possess a defective SAC will however proceed into anaphase with abnormal/misaligned chromosomes but should be eliminated at the G1 tetraploidy checkpoint. The tetraploidy checkpoint is thus the final barrier responsible for the elimination of tetraploid cells and it is probable that this checkpoint is not functional in the CT-1 cells since they have an average of 80 chromosomes per cell. The question thus arose as to how the CT-TBX2 cells have double this number of chromosomes and whether it is due to the over-expression of TBX2 compromising the SAC. This possibility was addressed by treating CT-E and CT-TBX2 cells with nocodazole, a microtubule depolymerising agent that activates the SAC, and then analysing the cells by flow cytometry.

In the presence of nocodazole, cells with a functional SAC arrest in metaphase and will only proceed through the cell cycle once the drug is removed. Cells that lack an intact SAC, however, do not arrest and proceed to cycle in the presence of the drug (Straight and Murray,

1997). Briefly, cells were treated with nocodazole for 16 h after which the drug was removed for 0, 1, 2, 4 and 8 h and their cell cycle profile was analysed by flow cytometry for each time point (Fig. 3.5). The results show that both the CT-E (4N) and CT-TBX2 cells (4N and 8N populations) undergo a G2/M arrest following nocodazole treatment (0 h), indicating that both cell lines have a functional SAC. Furthermore, both cell lines were released from this arrest at the same rate, with an emerging G1 population becoming evident 2 h after release from nocodazole. In addition, both cell lines displayed a cell cycle profile similar to that of an asynchronous population by 8 h. These results suggested that the increased ploidy and frequency of CIN in the CT-TBX2 cells was not due to TBX2 disabling the SAC.

It is important to note that the SAC does not recognise all errors in metaphase (Musacchio and Salmon, 2007). For example the SAC is unable to detect merotelic attachment, where chromatids are attached to both spindle poles, and this can result in the presence of anaphase lagging chromosomes (Musacchio and Salmon, 2007; Cimini, 2008). Furthermore, dicentric chromosomes, which do not impair correct kinetochore–microtubule attachment or tension during metaphase, present another error that may not be detected by the SAC and this can result in consequent anaphase bridges (described in 1.5.4) (Tan et al., 2005). The next set of experiments was therefore carried out to identify the possible presence of anaphase bridges and lagging chromosomes in the CT-TBX2 cells. This was achieved by examining anaphase and telophase chromosomes by immunofluorescence microscopy. Figure 3.6 shows examples of trailing/lagging chromosomes and chromatin bridges in between separating anaphase chromosome masses (anaphase bridges) which was seen in, on average, 46% of CT-TBX2 cells undergoing mitosis. These mitotic defects may be the reason for the initial cell cycle block seen in CT-TBX2 cells (see Fig. 3.3b) because anaphase bridges present a physical barrier to furrow formation and thus prevents successful cytokinesis. This result was confirmed by transiently expressing TBX2 in the cos-7 cell line which does not express endogenous TBX2 and again the resulting cells exhibited lagging chromosomes and anaphase bridges (data not shown).

A study by Bilican et al. (2006) demonstrated that Tbx2 protein levels are tightly regulated during the various phases of the cell cycle with the levels peaking at G2 and being greatly diminished in mitosis. It was therefore hypothesised in this thesis that in order to proceed through mitosis TBX2 needs to be degraded and that the mitotic defects observed in the CT-

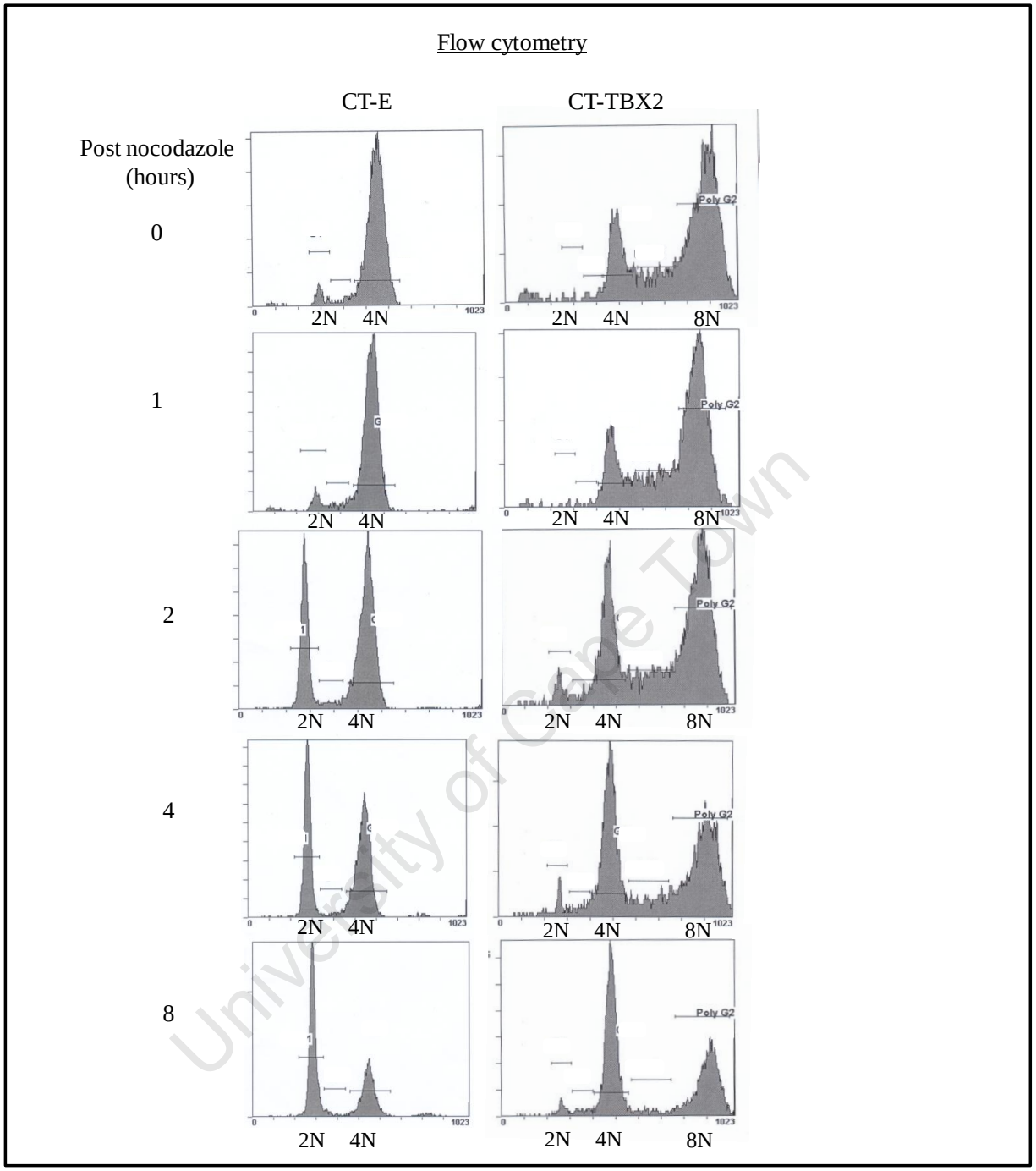

Figure 3.5. The CT-E and CT-TBX2 cells both display a functional spindle assembly checkpoint (SAC). Cells were arrested in metaphase following treatment with nocodazole (100 ng/ml) for 16 h and then released for 0, 1, 2, 4 and 8 h. The cells were stained with propidium iodide and the cell cycle distribution analysed by flow cytometry.

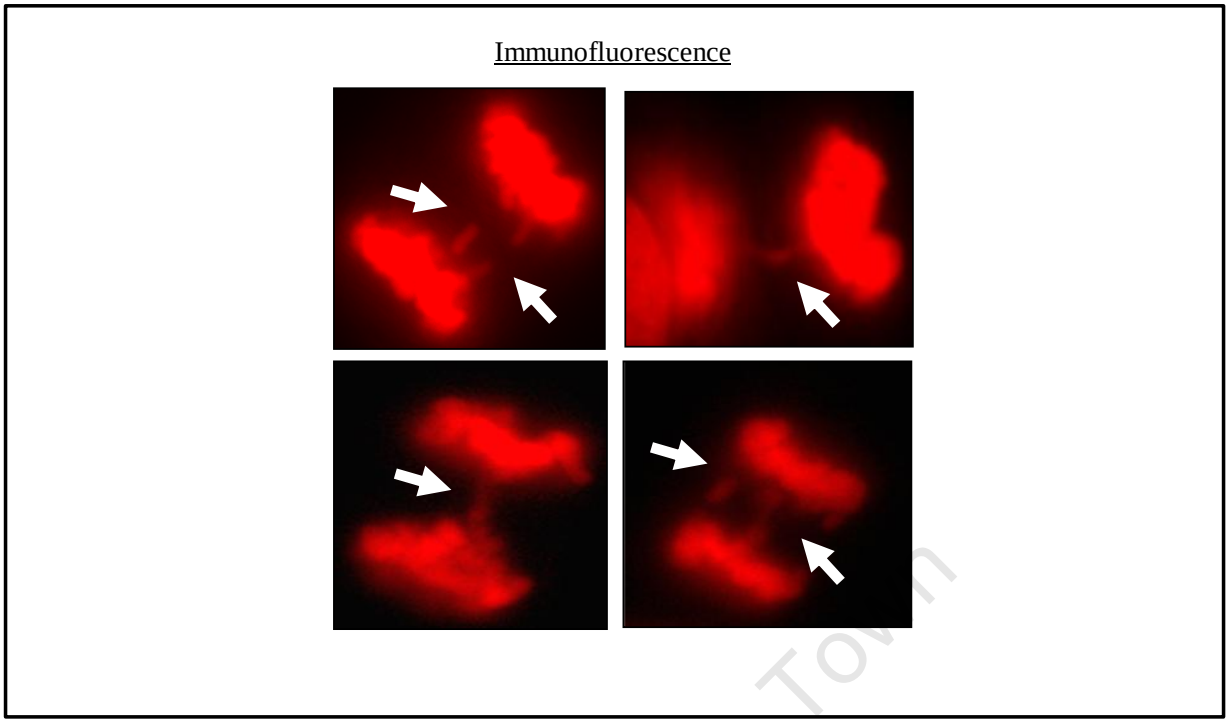

Figure 3.6. TBX2 over-expression induces anaphase bridges. CT-TBX2 cells in anaphase display trailing chromosomes and anaphase bridges. To determine the effect of TBX2 expression on chromosomal segregation the DNA of CT-E and CT-TBX2 cells was stained with propidium iodide and the cells viewed by immunofluorescence. Lagging chromosomes and anaphase bridges seen in CT-TBX2 cells are shown with arrows. Photographs taken at 100X magnification.

TBX2 cells may be as a result of over-expressed TBX2 escaping degradation. To test this hypothesis, CT-TBX2 cells were treated with drugs that block the cells at different stages of the cell cycle (see materials and methods) and both the phosphorylation status and total levels of TBX2 analysed by western blotting. The phosphorylation status of TBX2 was determined because previous work in our laboratory has shown that TBX2 is multiply phosphorylated and that phosphorylation plays an important role in regulating its function (Abrahams et al., 2008). The cell cycle phase distribution was firstly assessed by measuring DNA content using flow cytometry. The data shown in figure 3.7(a) confirmed that the cells were partially synchronised in the appropriate phases and these conditions were therefore used for future experiments. Figure 3.7(b) shows that whereas levels of the two bands, representing hypo- and hyper-phosphorylated TBX2, were equal in G1/S and S, they were substantially elevated in G2 and persisted in M phase. Unlike the results obtained by Bilican et al. (2006), TBX2 protein levels in CT-TBX2 cells remains high in M phase. It is therefore possible that when TBX2 is either ectopically expressed or over-expressed as is the case in some cancers, the mechanism by which the protein is degraded after G2 is compromised and hence the protein interferes with mitotic events. This possibility may account for the lagging chromosomes and anaphase bridges seen in CT-TBX2 cells. It is however important to note that in the study by Bilican and colleagues (2006) the B16 mouse melanoma cells where used, whereas the CT-TBX2 cells used in the current study are human fibroblasts. One cannot therefore rule out the possibility that the differences between the results from this study and that of Bilican et al.'s could be due to differential regulation of TBX2 in different species or cell types.

Interestingly, the subcellular localisation of TBX2 was also found to be regulated during the cell cycle in the CT-TBX2 cells. Figure 3.8(a) shows that when CT-TBX2 cells were synchronised as above, TBX2 was exclusively cytoplasmic in asynchronous and G1/S arrested cells. In S-phase however the protein was either cytoplasmic or nuclear but never both. Importantly, almost all cells blocked in G2 exhibited only nuclear staining for TBX2. To confirm that this result was not due to the chemicals used to synchronise the cells, immunofluorescence was performed on asynchronous cells using anti-TBX2 and co-stained with an antibody specific for Ser10-phosphorylated histone H3. Phosphorylation of H3 Ser10 occurs in late G2, initiating primarily within pericentric heterochromatin and spreading as

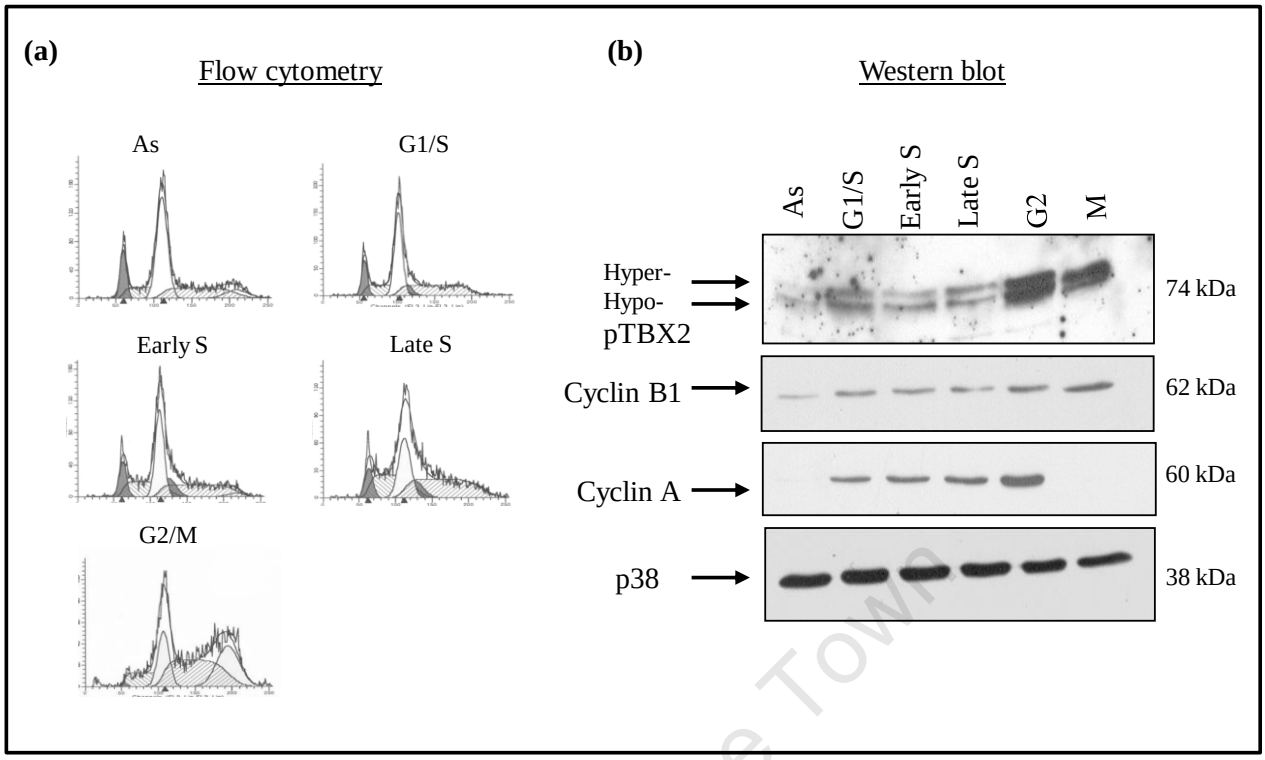

Figure 3.7. TBX2 protein levels persist in M-phase in CT-TBX2 cells. CT-TBX2 cells were arrested in G1/S by double thymidine block, released into S and treated for 16 h with nocodazole to obtain the G2 and M population of cells. M cells were collected by mitotic shake-off and the remaining attached cells were designated G2. These synchronised cells were analysed as described in (a) and (b). (a) Cell cycle distribution of asynchronous (as) or synchronised cultures of CT-TBX2 cells in specific stages of the cell cycle using flow cytometry. (b) Both the phosphorylation status and levels of TBX2 (hyper- and hypo- pTBX2) in CT-TBX2 cells are regulated in a cell cycle dependent manner. Protein extracts (40 µg) from both asynchronous or synchronised CT-TBX2 cells were analysed by 7.5-12% SDS-PAGE and western blotting to detect levels of phosphorylated TBX2, cyclin B1 and cyclin A. Anti-p38 antibody was used as a loading control.

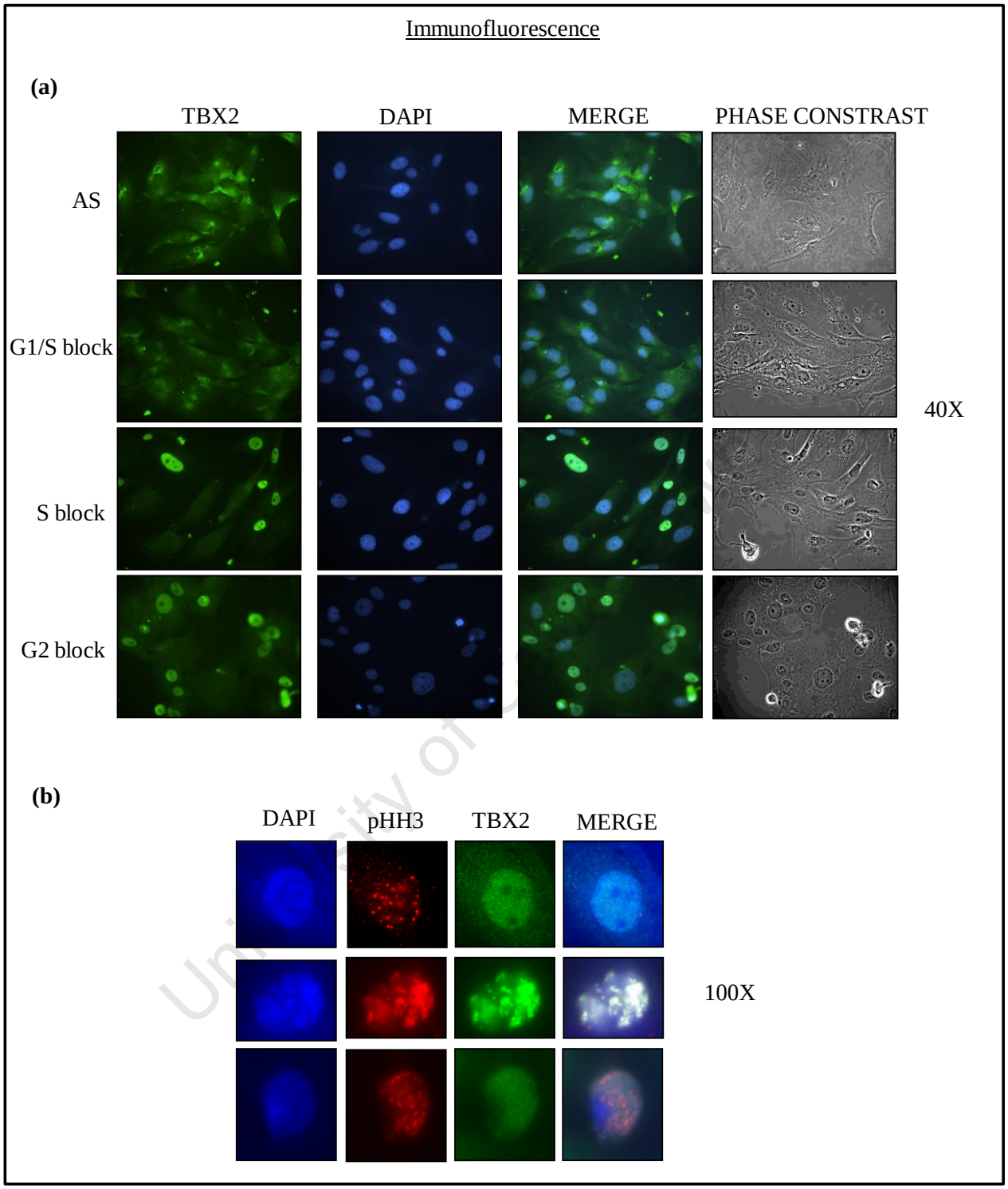

Figure 3.8. TBX2 localises to the nucleus in the CT-TBX2 cells going through G2. (a) Cells were synchronised as described in figure 3.7 and visualised by immunofluorescence using an antibody to TBX2. The far right hand side panel shows the corresponding phase contrast images. Images were captured at 40X magnification. (b) TBX2 co-localises with phospho-histone H3 (pHH3) in the nuclei of G2 cells. Asynchronous CT-TBX2 cells were stained with TBX2 and pHH3 which generates a specific punctate pattern of staining during G2. Images were captured at 100X magnification. All cells were stained with DAPI to determine the location of the nuclei.

chromatin condenses prior to mitosis (Hendzel et al., 1997). The results confirmed that TBX2 expression was nuclear in cells that also exhibited a punctate pattern of staining for phosphorylated H3 which is typical for G2 cells (Fig. 3.8b). Taken together the results show that increased protein levels correlate with increased nuclear localisation at G2, and therefore may suggest that TBX2 has a function in G2.

3.1.5 TBX2 over-expression resulted in cisplatin resistance

Reports have shown a relationship between genetic alterations in tumours, such as gene amplification and polyploidy, and the development of resistance to chemotherapeutic agents (Rothenberg and Ling, 1989; Baroja et al., 1998). We therefore compared the effect of the anti-cancer drug, cisplatin, on the growth rate of the CT-TBX2 and CT-E cells. The cells were cultured for 72 h, either in the presence or absence of cisplatin, and cell viability measured using the MTT assay. As shown in figure 3.9(a), while 50 µM cisplatin killed all of the CT-E cells, approximately 15% of CT-TBX2 cells (as compared to untreated cells) continued to survive. Importantly, even at 166.7 µM of cisplatin approximately 8% of CT-TBX2 cells were still viable (results not shown). Fifty percent growth inhibition (IC_{50}) in two CT-TBX2 clones and one CT-E clone was noted at concentrations of 17, 16 and 12 µM cisplatin, respectively (Fig. 3.9b). To exclude the possibility that the apparent increased resistance of CT-TBX2 to cisplatin was due to a percentage of the cells not dividing, BrdU incorporation assays were carried out. The cells were grown with or without 50 µM cisplatin for 48 h in the presence of BrdU and processed for BrdU incorporation by microscopy. Figure 3.9(c) shows that in the absence of the drug all of the CT-E and CT-TBX2 cells have incorporated BrdU and have therefore divided at least once. Furthermore, in the presence of 50 µM cisplatin there are considerably more viable BrdU positive CT-TBX2 cells compared to the CT-E cells (Fig. 3.9d). These results confirmed that the CT-TBX2 cells that survive cisplatin treatment are indeed dividing. Taken together, the results show that TBX2-expressing cells are more resistant to the anti-cancer drug cisplatin than the control cells.

In summary, the above data show that compared to their parental cells, the CT-TBX2 cells are larger, with binucleate and lobular nuclei containing double the number of chromosomes. Moreover, these cells had an increase in frequency of several features of genomic instability such as chromosome missegregation, chromosomal rearrangements and polyploidy. While grossly abnormal, these cells were still able to divide and gave rise to cells that were resistant

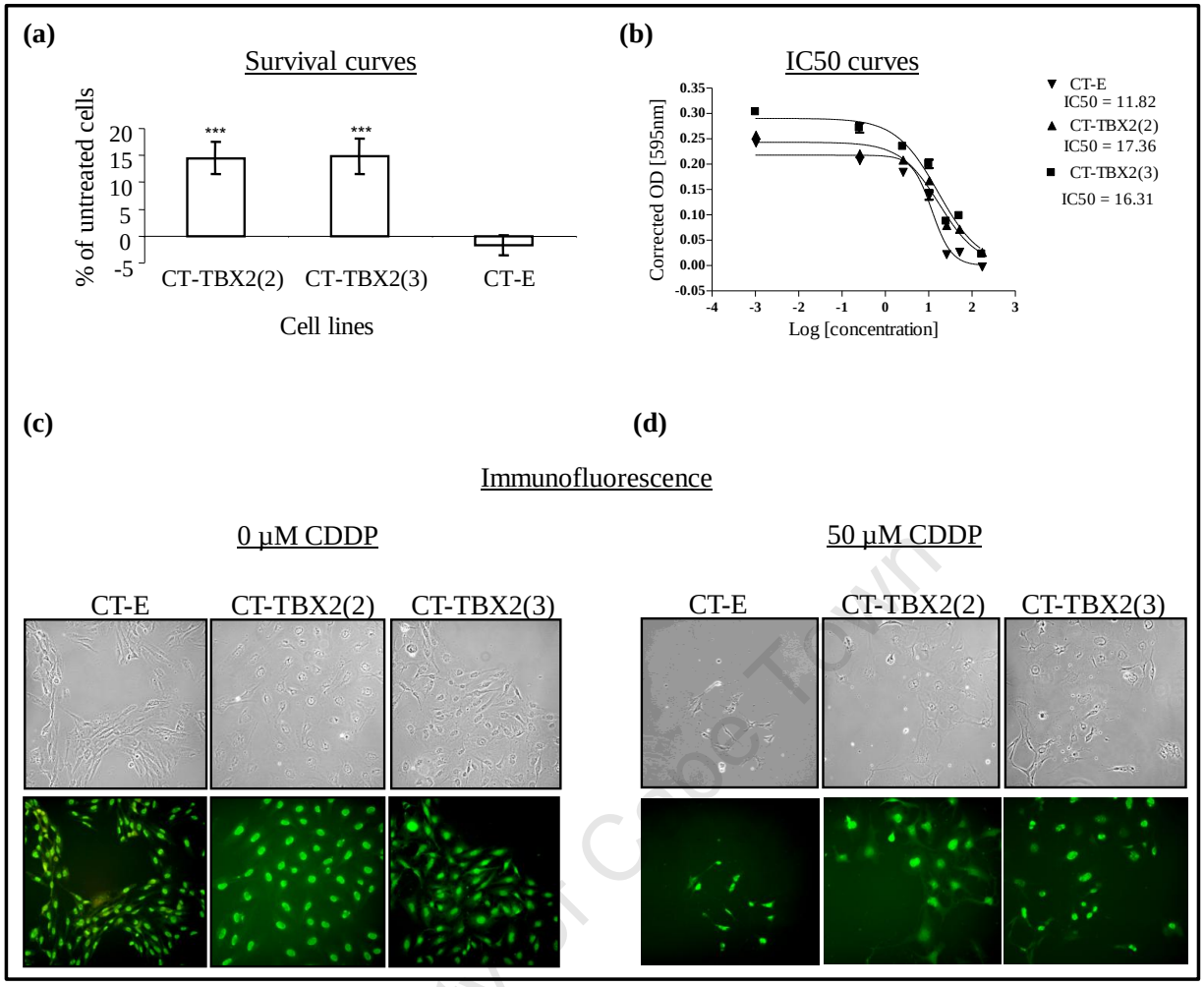

Figure 3.9. CT-TBX2 cells have increased resistance to the chemotherapeutic drug cisplatin. (a and b) CT-TBX2 cells have a higher IC50 than CT-E cells. Two CT-TBX2 clones and a CT-E cell line were seeded in triplicate at a density of $1X10^3$ cells per well of a 96-well plate. Cells were incubated with increasing concentrations of cisplatin (CDDP) for 72 h and cell viability determined using the MTT cell proliferation assay. (a) Cell survival, expressed as a percentage of untreated cells, when incubated with 50 µM cisplatin. The percentage survival for both CT-TBX2 cell lines were statistically significantly more as compared to the CT-E cell line (Student's unpaired t test, two-tailed, $p < 0.0001$). The result shown is representative of two independent experiments. (b) IC50 curves showing 50% growth inhibition in the indicated cell lines. (c and d) Cell survival is not due to cells not proliferating in the presence of cisplatin. Cells were grown in the presence of BrdU and 0 or 50 µM cisplatin for 48 h. Cells were harvested for immunocytochemistry using an anti-BrdU antibody. Fluorescence microscopy shows that (c) in the absence of the drug all CT-E and CT-TBX2 cells incorporate BrdU and (d) after treatment with 50 µM cisplatin substantially more CT-TBX2 cells are viable compared to control cells. Corresponding phase-contrast images are shown in the top panels. Photographs taken at 20X magnification.

to the chemotherapeutic drug cisplatin. Furthermore this was shown to be neither species nor cell type dependent, as ectopically expressing Tbx2 in a murine melanoma cell line also induced mitotic defects and polyploidy. The above results have important implications for our understanding of the role of TBX2 in tumourigenesis because polyploidy frequently precedes aneuploidy which is associated with high malignancy and poor prognosis.

3.2 *Silencing TBX2 expression in MCF-7 breast cancer and 501 melanoma cells*

One of the best approaches to understanding the function of a protein is to inhibit or reduce its expression and study the consequences on the cell or organism. In recent years, RNA interference (RNAi), and in particular small interfering (si)RNA which specifically target mRNAs containing a homologous sequence for degradation, has become the preferred technology for achieving this (as reviewed by Bantounas et al., 2004). This approach was therefore used in this study to further investigate the role of TBX2 over-expression in the oncogenic process. A summary of the cellular mechanism of gene silencing by RNAi is briefly described in the materials and methods.

One of the limitations of using siRNA technology is that the resultant knockdown is only transient and therefore repeated transfections are required for long term analysis. Sustained knockdown can be achieved through the use of small hairpin (sh)RNA, where an expression vector containing a polymerase III promoter can drive the synthesis of siRNAs. Briefly, the coding sequences for the sense and antisense strands of the targeted sequence were separated by a spacer region and cloned downstream of an appropriate polymerase III promoter, such as the H1 and U6 Polymerase III promoters. Once transcribed, the sense and antisense sequences will anneal to form double stranded RNA, and with the spacer region the resulting conformation is a hairpin or stem-loop structure (see figure 2.2). A stem-loop precursor is necessary to ensure the formation of the double stranded RNA which will be cleaved in the cell to produce a functional siRNA (Brummelkamp et al., 2002b).

The MCF-7 human breast adenocarcinoma cells, which express high levels of TBX2 (Jacobs et al., 2000), were chosen to stably knock down TBX2. This was achieved by stably transfecting the cells with a pSuper.neo/GFP vector expressing sequences targeting TBX2 or

a non-specific control sequence. The efficacy of TBX2 knockdown by four sequences was checked in transient transfections and the sequence which gave the most consistent silencing of TBX2 over a range of concentrations was selected for cloning into the pSuper.neo/GFP expression vector (data not shown). In order to facilitate cloning downstream of a polymerase-III H1-RNA gene promoter the sequences were designed with *Bgl*II and *Hin*dIII restriction enzyme sites at their 5' and 3' ends respectively.

Once transfected cells were grown in the presence of 400 µg/ml G418 and several drug resistant colonies were obtained and expanded into cell lines. The efficacy of the shRNA-mediated inhibition of TBX2 expression was initially monitored by western blotting using an anti-TBX2 antibody and although several clones were isolated all exhibited a similar phenotype and the results obtained for two are presented. Figure 3.10(a) shows that whereas TBX2 is undetectable in clones (shTBX2(3) and (5)), control cells (shcontrol(5) and (8)) have levels similar to that of the parental MCF-7 cells. These shTBX2 and shcontrol cell lines were therefore used for further analyses.

3.2.1 Knocking down TBX2 in MCF-7 breast cancer cells reduced the proliferation rate and re-established the requirement for mitogenic stimuli and a substrate

One of the defining characteristics of cancer cells is their ability to proliferate in an uncontrolled fashion and the first set of experiments thus investigated the effect of knocking down TBX2 protein levels on cell proliferation. Growth curve analyses were performed over 10 days and cell numbers determined using a haemocytometer. The shTBX2(3) and shTBX2(5) cell lines had a substantially reduced proliferation rate (Fig. 3.10b), growing approximately 4 and 8.5 times respectively more slowly on day 10, than the shcontrol(5) cells. To confirm the effect of knocking down TBX2 on cell proliferation BrdU incorporation assays were performed in which cells were pulsed with BrdU for 6 and 12 h. Figure 3.10(c) shows the total number of BrdU positive cells, expressed as a percentage of the total number of cells from 10 fields of view, for each cell line. At both time points tested, the shcontrol cells incorporated more BrdU compared to the shTBX2 cell lines and this difference was particularly significant after 12 h of incubation with BrdU. These results thus confirmed that knocking down TBX2 levels severely inhibit cell proliferation of the MCF-7 cells.

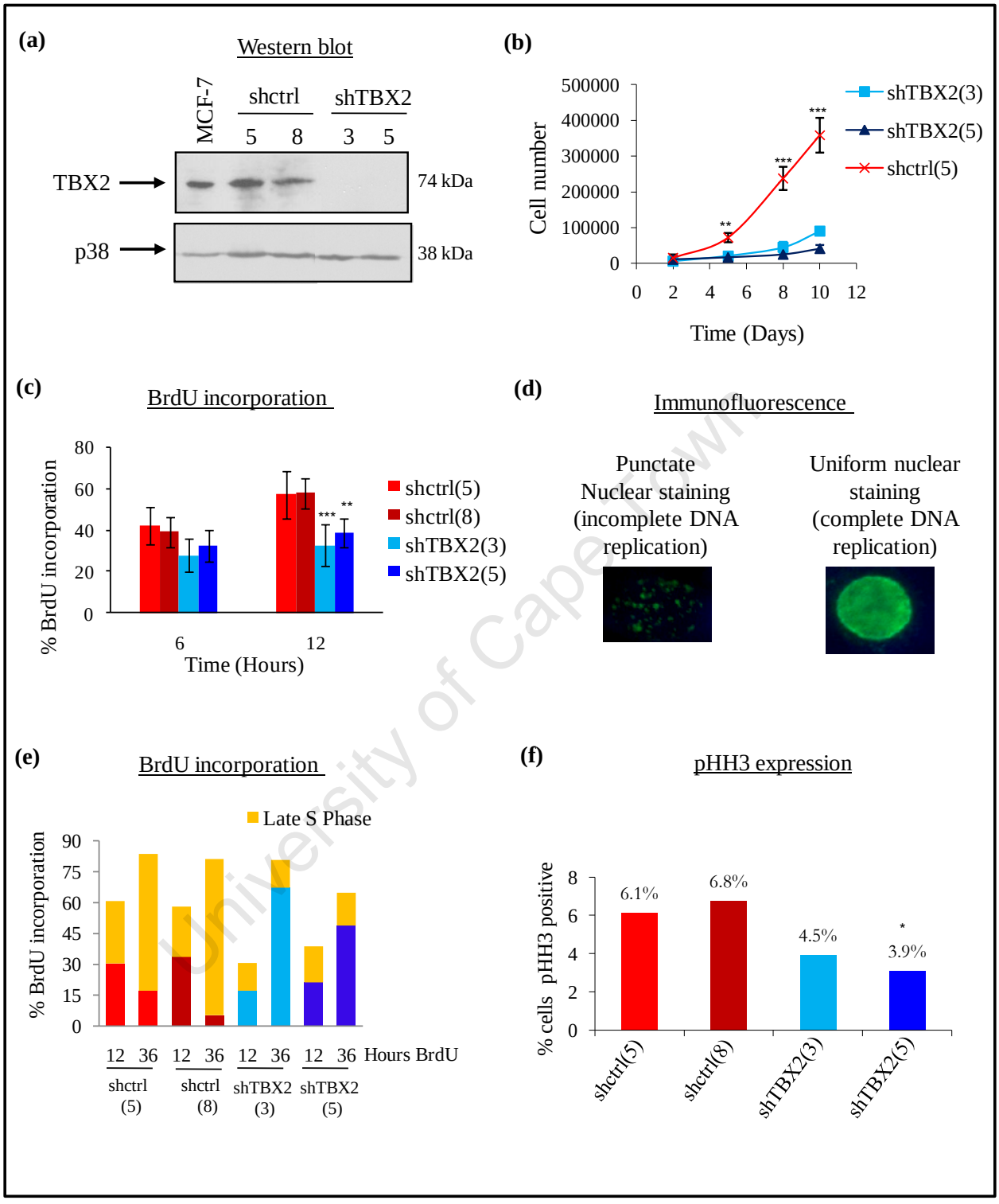

Fig. 3.10

Figure 3.10 Stable knockdown of TBX2 in MCF-7 cells decreases cell proliferation. Cells were stably transfected with the pSuper.neo/GFP (Oligoengine) expression vector containing either a TBX2 specific siRNA sequence (MCF-7 shTBX2) or a non-specific, scrambled control sequence (MCF-7 shctrl). (a) TBX2 is successfully knocked down in MCF-7 shTBX2 clones. Protein extracts (40 µg) harvested from the indicated cell lines were analysed by SDS-PAGE (8%) and western blotting using an anti-TBX2 antibody. p38 was used as a loading control. (b), (c) and (d) shTBX2 cells have reduced proliferative ability. (b) Cells were seeded in triplicate at a density of $1X10^4$ cells per well of a 12-well plate. Growth curve assays were performed over a 10-day period and cells harvested by trypsinisation and counted on a haemocytometer. The number of cells for both shTBX2 cell lines were statistically significantly less as compared to the shcontrol cell line on day 5, 8 and 10 (Student's unpaired t test, two-tailed, $p < 0.003$). The result shown is representative of three independent experiments. (c) Cells were seeded on sterile coverslips, pulsed with BrdU for 6 and 12 h and harvested for immunocytochemistry using an anti-BrdU antibody. BrdU-positive nuclei were visualised by fluorescence microscopy. Results show an average of 10 fields of view with BrdU-positive cells being expressed as a percentage of total cells counted. The percentage BrdU incorporation for both shTBX2 cell lines was statistically significantly less as compared to the shcontrol cell lines at 12h (Student's unpaired t test, two-tailed, $p < 0.002$). (d) Immunofluorescence images show examples of punctate and whole nuclear BrdU incorporation. (e) The percentage of cells that display punctate (incomplete replication, lower portion of each bar graph) and whole (complete replication, upper portion of each bar graph) nuclear BrdU staining was determined after 12 and 36 h of BrdU incorporation. (f) Fewer shTBX2 cells exit S-phase. The number of cells in G2 and M was determined by staining with an antibody to phospho-Histone H3 (pHH3) (ser-10) and viewed by fluorescence microscopy. Results show an average of 10 fields of view with pHH3-positive cells being expressed as a percentage of total cells counted. The number of pHH3 positive cells for the shTBX2(5) cell line was statistically significantly less as compared to the shcontrol cell lines (Student's unpaired t test, two-tailed, $p < 0.04$).

While counting the BrdU positive cells above, a very obvious difference in the staining pattern of the shcontrol and shTBX2 cells was observed. Whereas shTBX2 cells exhibited predominantly punctate nuclear staining, which indicated incomplete nuclear replication, the control cells had a more uniform nuclear stain, indicative of complete replication (examples shown in Fig. 3.10d). The observation was confirmed in figure 3.10(e) when the number of cells having punctate- (lower portion of each bar graph) compared to uniform- nuclear staining (upper, striped portion of each bar graph) was counted. At 12 h, approximately 50% of the shcontrol and shTBX2 BrdU positive cells display punctate nuclear staining. At 36 h however, approximately 90% of shcontrol cells, but only an average of 42% of shTBX2 cells, have uniform nuclear staining. These results suggest that the shTBX2 cells take a longer time to progress through S-phase which may reveal a possible role for TBX2 in S-phase.

To confirm the above result the number of shTBX2 and shcontrol cells progressing into G2 and M was determined. This was achieved by staining asynchronous cells for phospho-Histone H3 (pHH3) (ser-10) and counting the number of pHH3 positive (G2 and M) cells. As expected the total number of pHH3 positive cells was higher for shcontrol cells (6-7%) compared to the shTBX2 cells (3-4%) (Fig. 3.10f). This was not due to a block in G2 since the ratio of G2 to M cells was the same for the control and knockdown cells (data not shown). This confirmed that compared to shcontrol cells, fewer shTBX2 cells were progressing through S-phase. Furthermore, these results may provide a clue as to the basis for the reduced proliferative ability of the shTBX2 cells.

Transformed cells have reduced dependence on the levels of growth factors in their environment, therefore the ability of the shTBX2 and shcontrol cells to proliferate in low serum (2% FBS) medium, compared to normal growth medium containing 10% serum was investigated. As expected, when the growth rate of the shcontrol cells were compared in 2% versus 10% serum, there was no difference in the proliferation rate of the cells (Fig. 3.11a). In contrast, the shTBX2 cells cultured in 2% serum proliferated dramatically more slowly compared to their grown in 10% serum. Knocking down TBX2 levels in the MCF-7 cells therefore re-establishes their need for growth factors in their environment.

Another key characteristic of cancer cells is their ability to proliferate independently of a substrate. To determine whether TBX2 was required for anchorage independent growth of

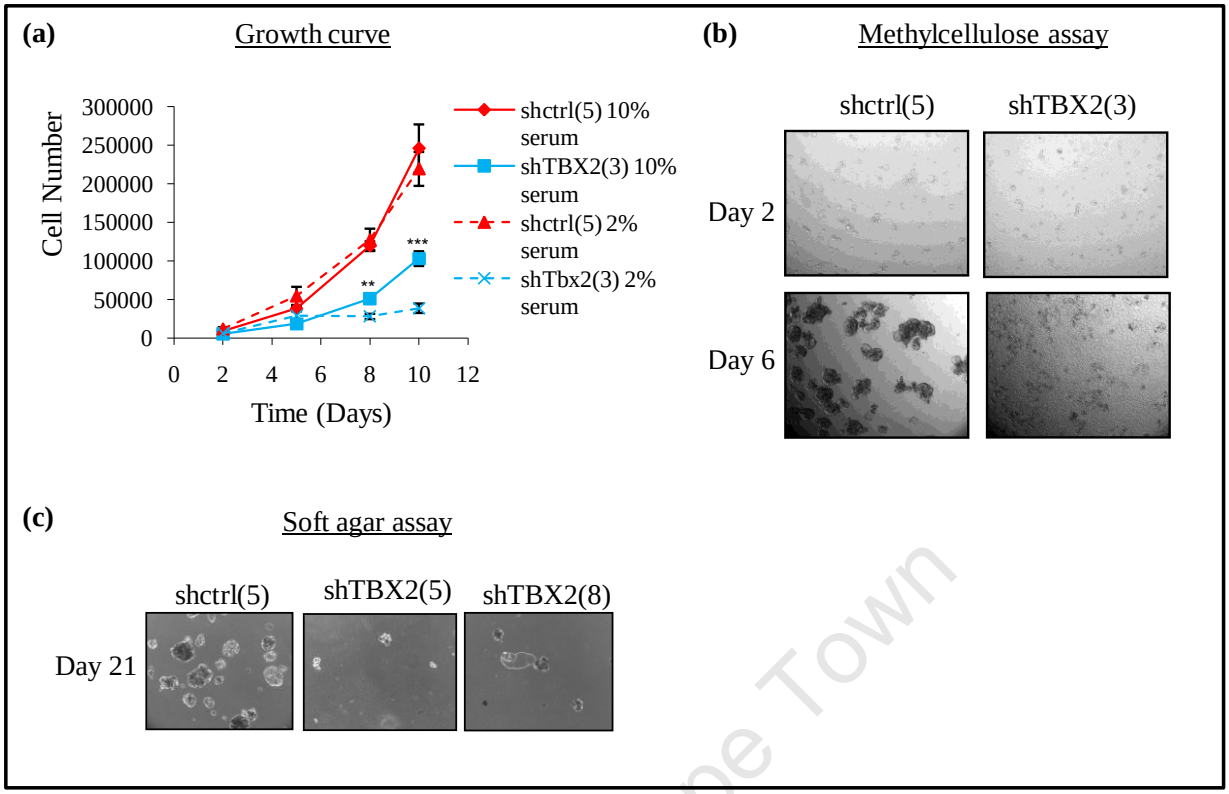

Figure 3.11. Knocking down TBX2 in MCF-7 cells re-establishes the requirement of the cells to proliferate in the presence of mitogenic stimuli and a substrate. (a) MCF-7 shTBX2 and shcontrol (shctrl) cells were grown in medium supplemented with either 10% or 2% FBS and growth curve assays were performed over a ten-day period. Cells were seeded in triplicate and counted on a haemocytometer. The number of cells for the shTBX2 cell line in 2% FBS were statistically significantly less as compared to that in 10% FBS on day 8 and 10 (Student's unpaired t test, two-tailed, $p < 0.002$). The above results represent one of two independent experiments. (b and c) Anchorage-independent growth of shTBX2 and shcontrol cells. (b) Cells were resuspended in 0.8% methylcellulose and plated into 96-well plates coated with polyHEMA. Cell growth was assessed over 7 days by staining with p-iodinitrotetrazolium-chloride to indicate viable populations and microscopic images were taken at 10X magnification. Day 2 images indicated that an equal number of cells was plated for all cell lines tested. (c) Cells were plated in soft agar and allowed to proliferate for 21 days. Images were captured at 10X magnification after 21 days of growth and show colony formation in the shcontrol and shTBX2 cell lines tested. All images taken using an Axiovert microscope (Zeiss, Germany).

MCF-7 cells, the ability of the shTBX2 and shcontrol cells to grow in suspension was assessed by seeding the cells in 0.8% methylcellulose and colony forming ability monitored over a 7 day period. Cells were stained with p-iodonitrotetrazolium-chloride to indicate viable populations and photographed at 10X magnification (Fig. 3.11b). Images taken 2 days after seeding the cells indicated that an equal number of cells were plated for both the shTBX2 and control cell lines. Whereas the shcontrol cells formed colonies by day 6, no colonies were observed for the shTBX2 cells. This result was confirmed by analysing the ability of the cells to form colonies in soft agar and monitoring colony formation over 3 weeks. Figure 3.11(c) shows that the shcontrol cells readily formed large viable colonies, unlike the shTBX2 cells which remained as predominantly single cells. Taken together, the above results convincingly show that knocking down TBX2 in MCF-7 cells re-establishes the requirement of the cells to proliferate in the presence of mitogenic stimuli and a substrate.

3.2.2 Suppression of TBX2 in melanoma cells also re-established the requirement for mitogenic stimuli and a substrate

The above results raised the question as to whether silencing TBX2 expression in another type of cancer would result in a similar phenotype as seen for the MCF-7 breast cancer cells. To this end the 501 human melanoma cells that express high levels of TBX2 (Vance et al., 2005) were stably transfected with the pSuper.neo/GFP (Oligoengine) expression vectors encoding either shTBX2 or shcontrol sequences as described for the MCF-7 cells. Following the generation of stable cell lines a number of G418 resistant clones were isolated and figure 3.12(a) shows that whereas TBX2 is undetectable in shTBX2 clones, control cells have levels similar to that of the parental 501 cells.

Experiments were performed as described for MCF-7 shTBX2 and shcontrol cells to determine the effect of knocking down TBX2 on key features of transformation in 501 melanoma cells. Results showed that the 501 shTBX2 cell lines had a much reduced proliferative ability compared to the 501 shcontrol cells, growing 3.7 times more slowly on day 9 (Fig. 3.12b). These results confirmed that silencing TBX2 expression in a different cancer also severely retards cell proliferation. The next set of experiments was performed to determine the impact of knocking down TBX2 in melanoma cells on growth factor independence. It is important to note that unlike most cancers, reports in the literature have shown that the growth of both poorly and highly metastatic melanoma cells is proportional to

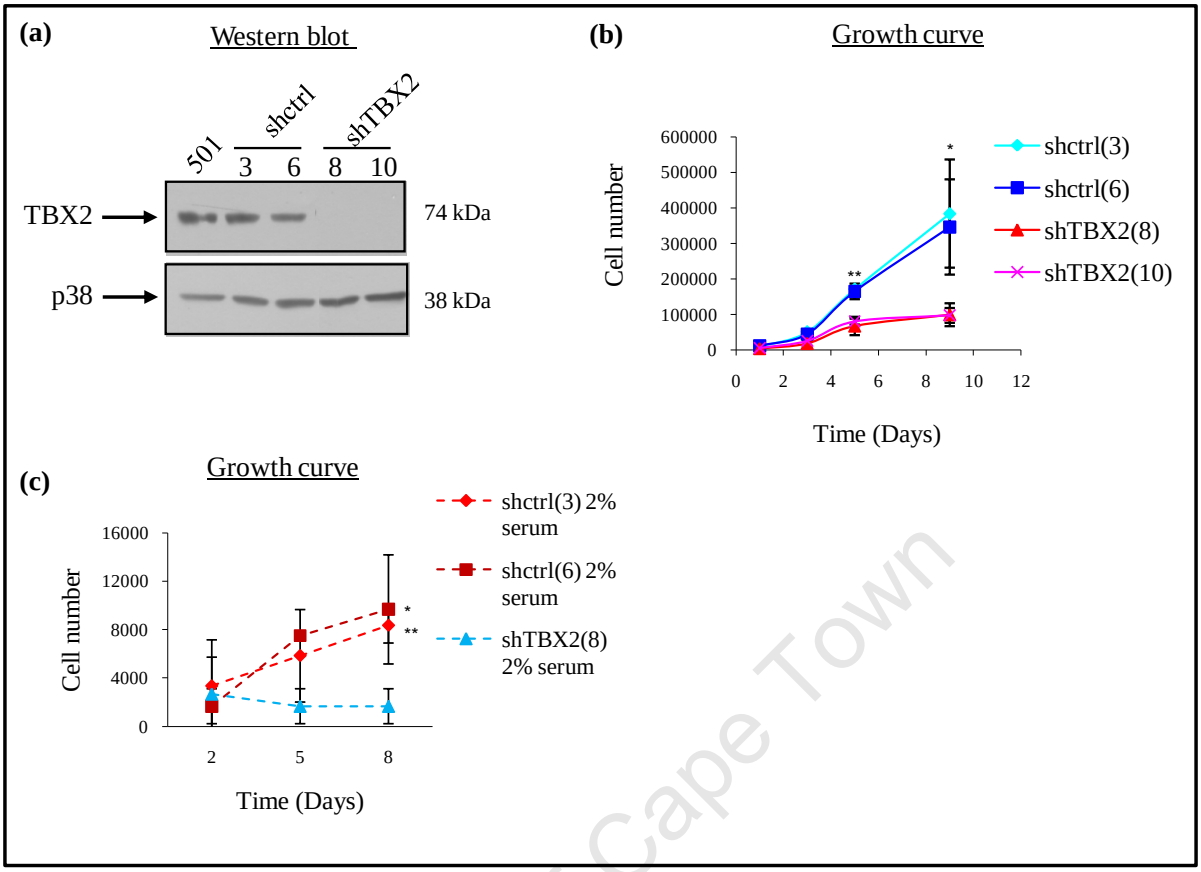

Figure 3.12. Decreasing TBX2 levels reduces the transforming potential of 501 melanoma cells. Cells were stably transfected with the pSuper.neo/GFP (Oligoengine) expression vector containing sequences that result in either effective knockdown of TBX2 (501 shTBX2) or a non-specific, scrambled control sequence (501 shctrl). (a) Western blot analysis shows effective knockdown of TBX2 protein levels in two of the shTBX2 clones that were used for subsequent experiments, as compared to that of two shcontrol clones. p38 levels indicate equal amounts of protein were present. (b) shTBX2 cells have reduced proliferative ability. Growth curve assays performed over a nine-day period, with cells seeded in triplicate and counted on a haemocytometer. The number of cells for the shTBX2 cell lines were statistically significantly less as compared to the shcontrol cell lines on day 5 and 9 (Student's unpaired t test, two-tailed, $p < 0.04$). Results shown represent one of three individual experiments. (c) shTBX2 and shcontrol cells were grown in medium supplemented with 2% FBS and growth curve assays were performed over an eight-day period. Cells were seeded in triplicate and counted on a haemocytometer. The number of cells for the shTBX2 cell line were statistically significantly less as compared to the shcontrol cell lines on day 8 (Student's unpaired t test, two-tailed, $p < 0.04$).

the levels of growth factors in their environment (Ellem and Kay, 1983; Mooradian, et al., 1990). This is indeed the case for 501 melanomas as the shcontrol cells grown in 2% serum had greatly reduced proliferative ability compared to cells grown in 10% serum (data not shown). To investigate the impact of knocking down TBX2 on growth factor dependence, cell growth were therefore compared in low serum (2% FBS) medium only. Figure 3.12(c) shows that unlike the shcontrol cells, the shTBX2 cells were not only unable to proliferate in low serum medium but they struggled to survive under these conditions. These results confirm that TBX2 has a profound effect on cell proliferation and their dependence on mitogenic stimuli.

To determine whether TBX2 was also required for anchorage independent growth of 501 melanoma cells, the ability of the shTBX2 and shcontrol cells was assessed using soft agar and methyl cellulose assays as above. As seen before for the MCF-7 cells, the 501 shTBX2 cells had considerably reduced colony-forming ability (Fig. 3.13). However, unlike the MCF-7 shTBX2 cells the 501 shTBX2 cells were able to form small colonies (Fig. 3.13c). The reason for this is discussed in chapter 4. Taken together, the above results confirm that silencing TBX2 in both a breast cancer and melanoma cell line reduces their transforming potential and that this is therefore not cell type dependent.

3.2.3 Knocking down TBX2 had no effect on the migration of 501 melanoma and MCF-7 breast cancer cells

In order for cancer cells to metastasise they must acquire the ability to invade adjacent tissues and establish colonies at new locations (Hanahan and Weinberg, 2000). To investigate whether knocking down TBX2 impacts on these later stages of tumourigenesis, the migration rate of the MCF-7 shcontrol and shTBX2 cell lines was compared. MCF-7 cells have poor invasive ability, but their migration can be induced by the addition of -*O*-tetradecanoylphorbol-13-acetate (TPA), a chemical that activates the protein kinase C pathway (Johnson et al., 1999). Previous work in our laboratory has shown that TPA induces TBX2 expression in human fibroblasts (Teng et al., 2009) and this raised the possibility that TBX2 may play a role in the TPA-induced migration of the MCF-7 cells. A two-dimensional *in vitro* scratch motility assay was carried out for both shcontrol and shTBX2 cells. Briefly, cells were grown to confluence and a linear wound was made by scratching through the monolayer using a sterile 200 µl pipette tip. To exclude the effect of cell proliferation on the

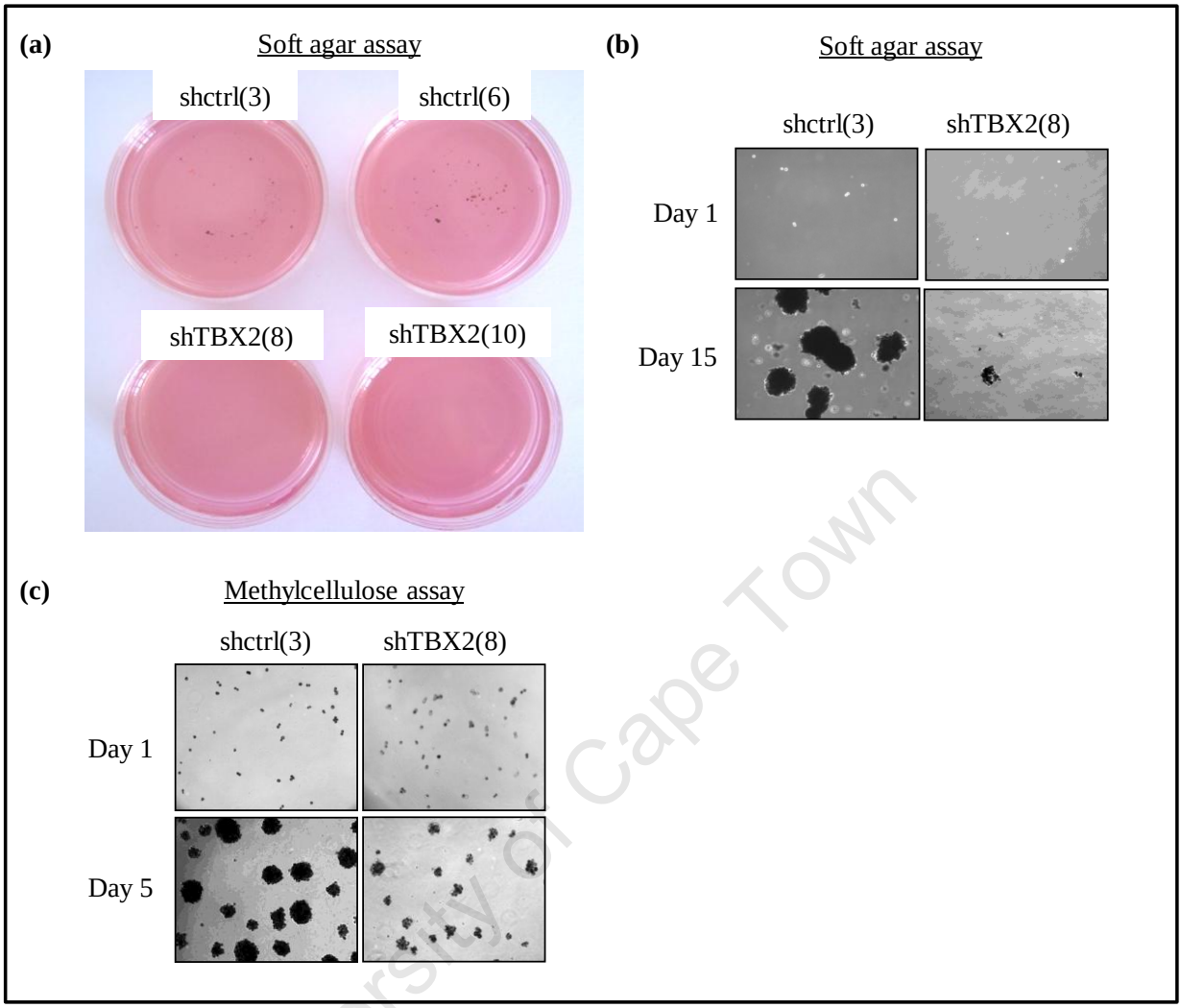

Figure 3.13. TBX2 promotes anchorage independent growth of 501 melanoma cells. (a and b) Cells were plated in soft agar and allowed to proliferate for 15 days. (a) Images of whole dishes at 15 days of proliferation. (b) Cell growth was assessed by staining with p-iodinitrotetrazolium-chloride to indicate viable populations. Microscopic images taken at 10X magnification show colony formation in the shcontrol (shctrl) and shTBX2 cell lines tested. Day 1 images (top panels) illustrate that equal numbers of cells were seeded. (c) Cells were resuspended in 0.8% methylcellulose, plated into 96-well plates coated with polyHEMA and cell growth assessed over 5 days. Day 1 images (top panels) indicated that an equal number of cells was plated for all cell lines tested. All microscopic images taken using an Axiovert microscope (Zeiss, Germany).

results, cells were treated with mitomycin C before TPA treatment. Figure 3.14(a) shows phase contrast images of the cell monolayer at 0 and 8 h after the scratch was made. The distance migrated between pre-marked points along the scratch was measured using Axio software over 8 h and the results revealed no significant difference between the migration rate of the shcontrol compared to that of the shTBX2 cells (Figure 3.14b). The same result was obtained for the 501 cells, which migrate independently of TPA (Fig. 3.14c). In this assay the results of two control cell lines were included to demonstrate that although the error bars show a large degree of variability, it was reproducible. These experiments showed that TBX2 had no effect on the migratory ability of breast cancer and melanoma cells tested in this study.

The results for the knock down experiments described above raised the interesting possibility that while TBX2 is a powerful pro-proliferative factor it does not contribute to the invasive cancer phenotype. The ability of the MCF-7- and 501- shTBX2 and shcontrol cells to form tumours in nude mice was therefore determined. Cells were injected into the flanks of nude mice and tumour growth monitored over a period of time. While no tumours were observed at the site of injection for either shcontrol or shTBX2 the lymph nodes of some mice became enlarged and firm to the touch which suggested that they harboured tumours and the size and the mass of these lymph nodes were thus recorded (Table 1). Statistical analyses on these data revealed that these differences were not statistically significant (unpaired t-test, 95% confidence interval). To ascertain whether the enlarged lymph nodes did indeed harbour tumour cells they were prepared for histological analysis and stained with haematoxylin and eosin. Light microscopic analyses show that lymph nodes from mice injected with either shcontrol or shTBX2 cells had no invading cancer cells. The enlarged lymph nodes however appear to be due to a dermatopathic lymphadenopathy (as suggested by the presence of large numbers of dendritic cells) for both shTBX2 and shcontrol cells (data not shown). These results therefore suggest that neither the MCF-7 nor 501 cells were able to form tumours under our experimental conditions. The effect of knocking down TBX2 on tumour forming ability *in vivo* is therefore inconclusive. It is important to note that the MCF-7 breast cancer cells are oestrogen dependent and even though female nude mice were used in this study, future experiments would probably need to be performed with exogenous oestrogen to induce tumour formation *in vivo*. In light of the effect of knocking down TBX2 on cell proliferation and anchorage independence it will be important to repeat these experiments.

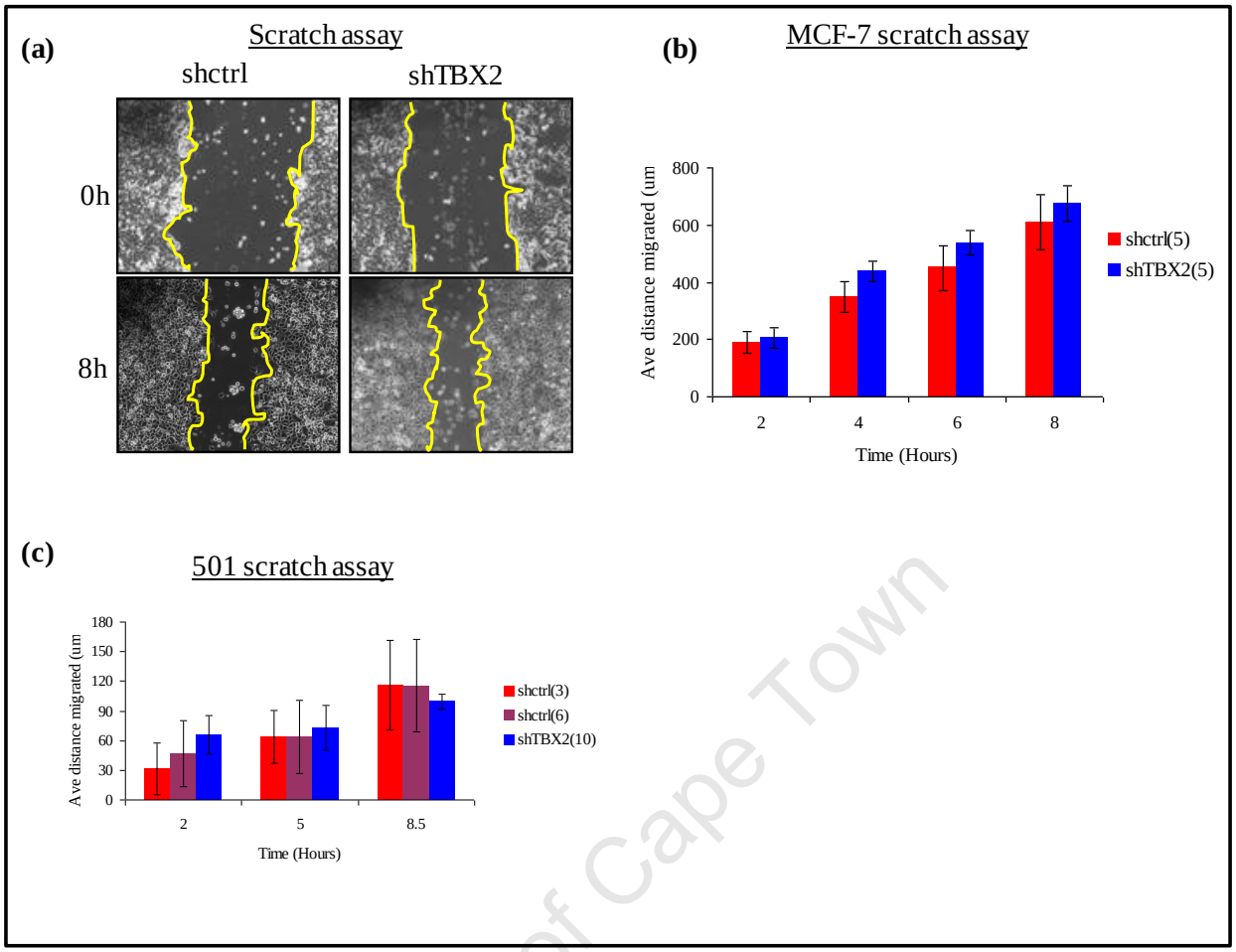

Figure 3.14. Knocking down TBX2 has no effect on the migratory ability of MCF-7 breast cancer and 501 melanoma cells. (a - c) shTBX2 and shcontrol (shctrl) cells have similar migratory ability. Migration was measured using a two-dimensional *in vitro* scratch motility assay. Cells were grown to confluence and a linear wound was made by scratching through the monolayer using a sterile 200 µl pipette tip. Mitomycin C (10 µg/ml) was added to prevent cell proliferation and migration measured at 1 – 2 h intervals. (a) Phase contrast images show the scratch line and 5 points along this line in different fields of view (therefore not visible) were used as reference points to measure the width, shown at 0 and 8 h after making the scratch (10X magnification). The yellow drawn in line indicates the width measured. Images were captured using a phase contrast microscope and migration distances measured using Axio software (Zeiss, Germany). (b) The average distance migrated by the MCF-7 shTBX2(5) and shcontrol(5) cell lines. MCF-7 cell migration was induced by adding TPA at a final concentration of 100 nM. (c) The average distance migrated by the 501 shTBX2(10) and two shcontrol cell lines.

Table 1. Enlarged lymph nodes in 6-8 week-old MF-1 nude mice injected with MCF-7 and 501 shTBX2 and shcontrol cells.

Cell line	Number of enlarged lymph nodes*	Ave. mass (g)	Ave. longest length (mm)
MCF-7 shcontrol[a]	3/6	0.219	4.85
MCF-7 shTBX2[a]	5/6	0.139	4.1
501 shcontrol[b]	7/7	0.0114	3.79
501 shTBX2[b]	6/7	0.0118	3.817

**Number of mice with enlarged lymph nodes/number of mice injected*

[a] $1X10^6$ cells injected and lymph nodes harvested 174 days post injection

[b] $2X10^6$ cells injected and lymph nodes harvested 129 days post injection

3.2.4 S-phase arrest induced by cisplatin in MCF-7 breast cancer and 501 melanoma cells required TBX2 expression

The data generated in the previous section suggested that abnormally high levels of TBX2 promote cell proliferation and that this may occur through its ability to compromise cell cycle checkpoints. The next set of experiments therefore analysed the integrity of cell cycle checkpoints in shcontrol and shTBX2 cells using the DNA damaging agent, cisplatin. Cisplatin, also referred to as cisplatinum or *cis*-diamminedichloroplatinum(II) (CDDP), is a platinum based compound that is able to bind DNA and form intrastrand and interstrand DNA adducts between purine residues (reviewed by Fuertes et al., 2003; Siddik, 2003; Torigoe et al., 2005). It usually elicits a transient S-phase arrest followed by a more sustained G2/M arrest (Siddik, 2003). MCF-7 cells were incubated with 10 μM cisplatin for 24, 48 and 72 h and their cell cycle distribution analysed by flow cytometry. Consistent with the published literature (Kobayashi et al., 1989; Baldassarre et al., 2005; Reinhardt et al., 2007), a large proportion of the shcontrol cells arrested in S-phase at all time points tested (Fig.3.15a, upper panel) and after 48 h a small population of cells progressed into G2/M where they arrested. Unlike the shcontrol cells, only a small population of shTBX2 cells arrested in S-phase at 24 h with the majority of the cells arresting in G2/M at 48 and 72 h (Fig.3.15a, bottom panel). The concentration of cisplatin used in this experiment was based on the published literature and similar results were obtained with 5 μM cisplatin (data not shown).

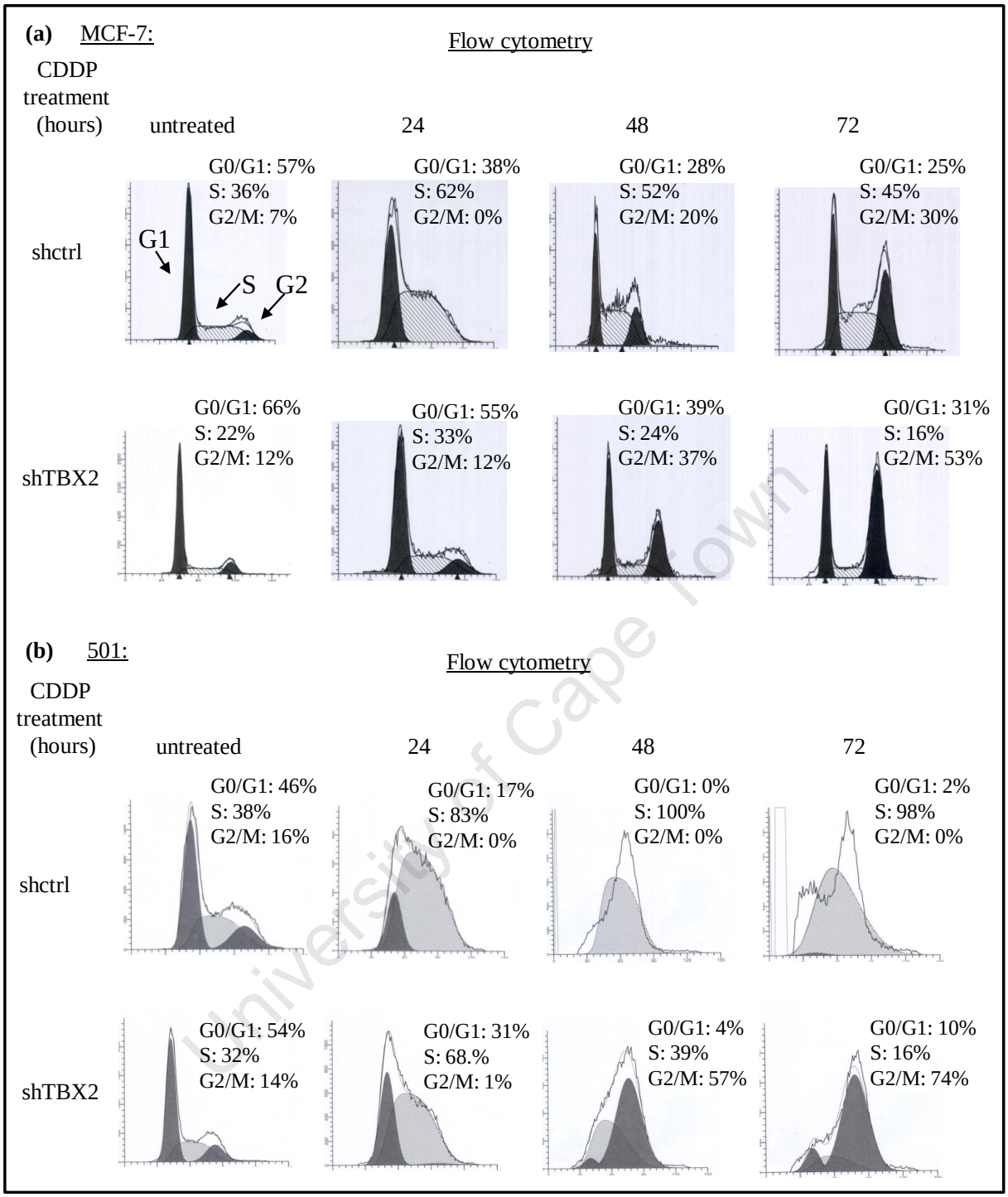

Figure 3.15. TBX2 is required for S-phase arrest following cisplatin treatment. (a) MCF-7 shcontrol (shctrl) (upper panel) and shTBX2 (lower panel) cells were treated with 10 µM cisplatin (CDDP) for 24, 48 and 72 h, their DNA stained with propidium iodide and analysed by flow cytometry. The proportion of cells at each phase of the cell cycle is presented as a percentage of the total number of cells analysed. (b) 501 shcontrol (upper panel) and shTBX2 (lower panel) cells were treated with 5 µM cisplatin for 24, 48 and 72 h and processed as described for (a).

This apparent abrogation of the S-phase arrest in MCF-7 shTBX2 cells was initially unexpected because it was anticipated that knocking down TBX2 would lead to a more robust activation of cell cycle checkpoints in response to DNA damage. Interestingly, when the above experiments were performed using 501 shcontrol and shTBX2 cells similar results were obtained (Fig. 3.15b). However, while the 501 shTBX2 cells did arrest in S-phase at 24 h, this arrest was not maintained and again the majority of the cells arrested in G2/M at 48 and 72 h (Fig. 3.15b, bottom panel). Although the shcontrol cells displayed a similar response to that observed for the MCF-7 control cells at 24 h, the S-phase arrest was maintained for the duration of the treatment (Fig. 3.15b, upper panel). In these experiments 5 μM cisplatin was used, as the 10 μM concentration was found to be too toxic for this cell type. Taken together the above data showed that while both the MCF-7 and 501 control cells preferentially arrested in S-phase in response to cisplatin treatment, the absence of TBX2 appeared to compromise this ability and favoured a G2/M arrest.

3.2.5 Knocking down TBX2 induced mitotic catastrophe after cisplatin-induced damage

As mentioned earlier, the above results appeared counter-intuitive because one might have expected the TBX2 knockdown cells to have a greater ability to arrest, for example, at the S-phase checkpoint. The recent literature however suggests that checkpoints, and in particular the S-phase checkpoint, may be beneficial to cancer cells because it enables them to repair damaged DNA induced by chemotherapeutic agents, leading to drug resistance (Levesque and Eastman, 2007; Levesque et al., 2008; Bucher and Britten, 2008). This raised the interesting possibility that knocking down TBX2 may sensitise MCF-7 breast cancer and 501 melanoma cells to cisplatin. The results obtained thus far were similar for MCF-7 and 501 cells and due to time constraints the next set of experiments were only performed in the MCF-7 cells. This was particularly relevant because the MCF-7 breast cancer cells have been reported to be resistant to cisplatin treatment (Fan et al., 1995; Yde and Issinger, 2006). It was therefore speculated that this may be through their ability to repair cisplatin-induced DNA damage by arresting in S-phase. If this were the case then the weakened S-phase arrest observed for the shTBX2 cells would force the cells to enter mitosis with damaged DNA which would lead to mitotic catastrophe.

Since mitotic catastrophe is characterised by mitotic cells with damaged DNA the next set of experiments examined cisplatin treated shcontrol and shTBX2 cells for the co-expression of mitotic (cyclin B1 and Ser10-phosphorylated histone H3 (pHH3)) and DNA damage (ser139-phosphorylated H2A.X (γ-H2AX)) markers. Cells were treated with 10 μM cisplatin as described before and figure 3.16(a) shows that the shTBX2 cells did indeed have elevated levels of cyclin B1 with correspondingly high increases in γ-H2AX in response to cisplatin. These results suggest that shTBX2 cells treated with cisplatin do undergo mitotic catastrophe and that the population of cells treated for 72 h (see Fig. 3.15a) are more likely to be cells in mitosis rather than G2 arrested cells. This is particularly interesting because the results obtained for the shcontrol cells suggested that they do not enter mitosis as their cyclin B1 and pHH3 levels decreased with cisplatin treatment (Fig. 3.16a). Furthermore, although an increase in γ-H2AX was observed in shcontrol cells at 24 and 48 h, it decreased at 72 h suggesting that they begin to repair the cisplatin-induced DNA damage at this time point. Interestingly, while the levels of pHH3 were high in shTBX2 untreated cells there was no consistent change in the levels of this protein in cisplatin treated cells. Although phosphorylation of HH3 at serine 10 is frequently used as a mitotic marker, this modification also occurs in other processes including during the transcription of a number of genes (Nowak and Corces, 2004) and therefore the levels of cyclin B1 was considered a more reliable marker of mitosis.

To confirm the above western blot results the MCF-7 shcontrol and shTBX2 cells were treated as above and processed for immunocytochemistry with an antibody to γ-H2AX. H2A.X is a variant form of histone H2A that is directly phosphorylated at Ser139 (γ-H2AX) in response to DNA double strand breaks and can be identified as discrete foci at the site of damage when stained by immunocytochemistry. This characteristic staining pattern marks an early event in response to DNA damage (Rogakou et al., 1998**).** Mitotic cells were identified by their characteristic DAPI staining (Fig. 1.16b, left most panel). As expected, both untreated shcontrol and shTBX2 cells had very few γ-H2AX foci and this was particularly true for cells undergoing mitosis. An example of this staining is shown in figure 3.16(b), top panel. Importantly, while γ-H2AX foci were present in both shcontrol and shTBX2 treated cells (data not shown), there were many more mitotic shTBX2 cells with γ-H2AX foci (Fig 3.16(b), bottom panel). Indeed, even though the same number of shcontrol and shTBX2 cells

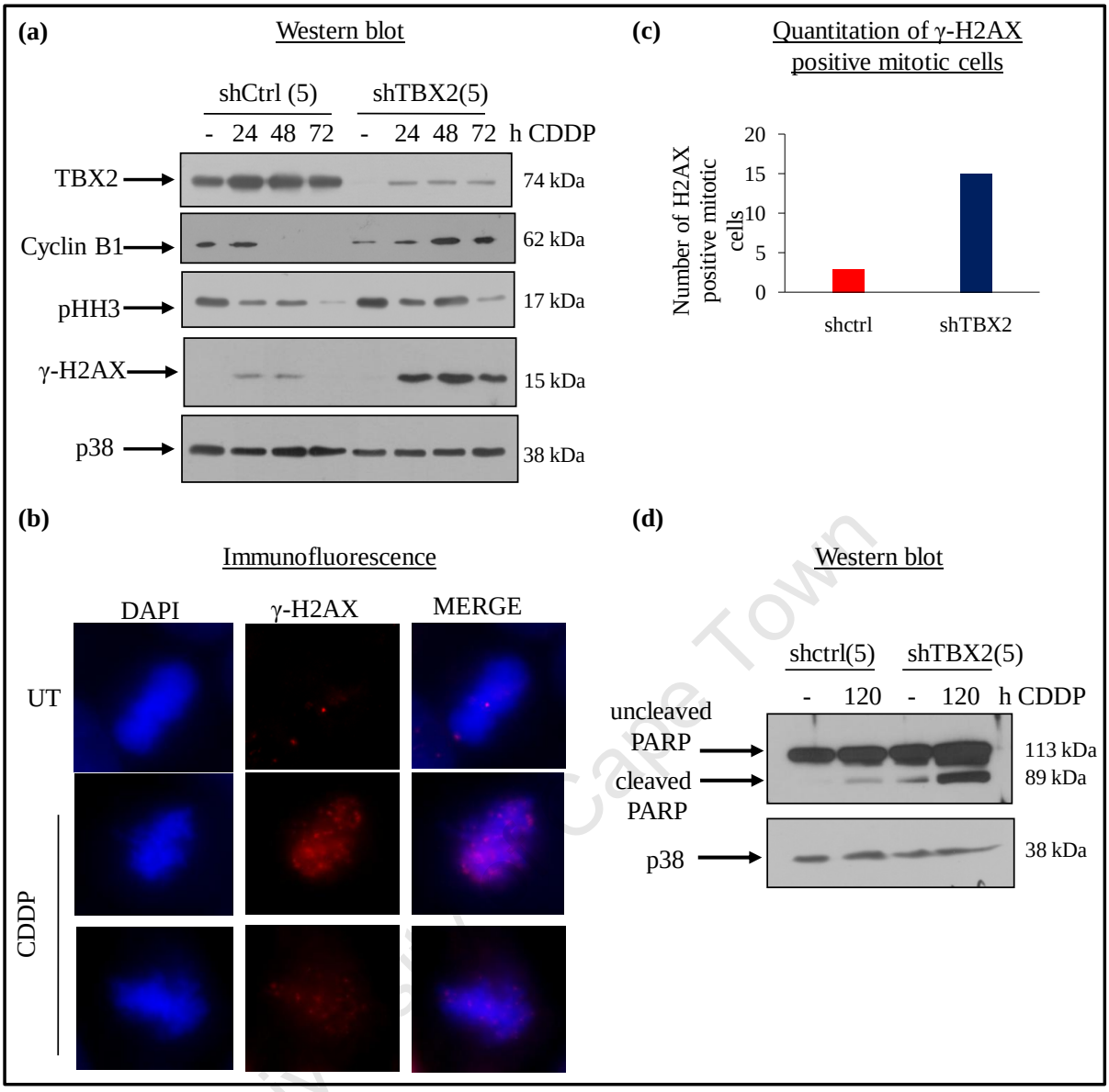

Figure 3.16. Knock down of TBX2 sensitises MCF-7 cells to cisplatin by inducing mitotic catastrophe. (a) shTBX2 cells have increased levels of cyclin B1 and γ-H2AX. MCF-7 shcontrol (shctrl) and shTBX2 cells were treated with 10 µM cisplatin (CDDP) for 24, 48 and 72 h and protein extracts (40 µg) were analysed by SDS-PAGE (8-15%) and western blotting using the indicated antibodies. p38 was used as a loading control. (b and c) More shTBX2 cells enter mitosis with DNA damage. An equal number of cells were plated on a sterile coverslip, treated with 10 µM cisplatin for 72 h and harvested for immunocytochemistry using an antibody to γ-H2AX and co-staining with DAPI to identify mitotic cells. (b) Fluorescence microscopy images of untreated and cisplatin treated shTBX2 cells stained with DAPI (to identify cells in mitosis) and anti-γ-H2AX. Images captured using an Axiovert fluorescent microscope (Zeiss, Germany) 100X magnification. (c) The total number of mitotic cells that were positive for γ-H2AX per coverslip. (d) shTBX2 cells have increased levels of uncleaved and cleaved poly(ADP-ribose) polymerase (PARP). Cells were treated with 10 µM CDDP for 120 h and protein extracts (30 µg) harvested from the indicated samples were analysed by SDS-PAGE (8%) and western blotting using an antibody that recognises uncleaved and cleaved PARP. Arrows indicate uncleaved (113 kDa) and cleaved (89 kDa) PARP. p38 was used as a loading control.

was treated with cisplatin, when all cells were analysed, 5 times more shTBX2 mitotic cells stained positively for γ-H2AX (Fig. 3.16c).

The above results suggested that the shTBX2 cells were unable to repair cisplatin-induced DNA damage and that these cells were consequently driven into mitotic catastrophe. Mitotic catastrophe occurs via the activation of apoptotic pathways involving a number of apoptotic proteins (Castedo et al., 2004). One such protein, poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme activated by DNA strand breaks and participates in DNA repair using nicotinamide adenine dinucleotide (NAD) as a substrate. However in the case of excessive DNA damage, rapid elevation of PARP results in the depletion of NAD which leads to cell dysfunction and programmed cell death (Berger, 1985; reviewed by Pieper et al., 1999). During apoptosis PARP is inactivated by cleavage into 89-and 24-kDa fragments (Kaufmann, 1989; Pieper et al., 1999) and thus the levels of both uncleaved and cleaved PARP are effective markers of apoptosis. The effect of knocking down TBX2 on an apoptotic marker was measured in cisplatin treated MCF-7 shcontrol and shTBX2 cells. The results showed a more dramatic increase in the levels of both uncleaved and cleaved PARP in the shTBX2 cells compared to the shcontrol cells when treated with cisplatin for 120 h (Fig. 3.16d). Furthermore, it is interesting to note that when comparing the levels of both uncleaved and cleaved PARP in untreated cells, they are elevated in the shTBX2 cells indicating that knocking down TBX2 either results in increased susceptibility to DNA damage or induces some form of cellular stress leading to the activation of apoptotic pathways. Taken together, these data show that knocking down TBX2 in the MCF-7 cells sensitises the cells to cisplatin treatment by preventing DNA damage repair in S-phase and promoting mitotic catastrophe.

3.2.6 TBX2 was required for p53 induction in MCF-7 cells following cisplatin treatment

The next set of experiments was carried out to begin to understand the molecular mechanism regulating the effect of TBX2 depletion on the cisplatin response in MCF-7 cells. This was regarded as an important approach because it could provide clues as to the "normal" role of TBX2 in the DNA damage response. Furthermore, this could reveal potential ways of interfering with the role of TBX2 in conferring cisplatin resistance in MCF-7 cells. The levels of TBX2 protein following cisplatin treatment were firstly examined by western blot analysis and figure 3.17(a) shows TBX2 levels increased in the shcontrol cells at all time points

tested. Interestingly, while TBX2 was undetectable in the untreated shTBX2 cells, the protein was present at low levels in all cisplatin treated shTBX2 cells. One possibility for this observation could be that TBX2 mRNA levels increased due to either transcription or posttranscriptional stabilization of the mRNA and the elevated levels allowed some TBX2 mRNA to escape the siRNA machinery. Another possibility is that because siRNA is not 100% efficient, low levels of TBX2 protein is posttranslationaly modified and stabilised upon cisplatin treatment. The latter possibility is particularly attractive because previous work in our laboratory has shown that in response to UV stress the TBX2 protein is phosphorylated by the p38 MAP kinase and that this led to an increase in TBX2 protein stability (Abrahams et al., 2008). The increase in TBX2 levels in response to cisplatin-induced damage indicated that TBX2 may play a role in the DNA damage response.

A number of pathways have been implicated in the cisplatin-induced DNA damage response (Fig. 3.18) (Siddik, 2003; Torigoe et al., 2005). Most of these pathways in turn activate p53, which transcriptionally activates downstream targets such as *p21*, which maintains the cell cycle arrest, *Gadd45a*, which enhances nucleotide excision repair, or pro-apoptotic factors such as *bax* and the caspase 8-caspase 3 pathway (Fuertes et al., 2003; Siddik, 2003). The factors initiating repair pathways include ATM and ATR which frequently control DNA damage checkpoints in mammalian cells (Hakem, 2008). The levels of total ATM and ATR were therefore determined by treating the cells as before and analysing the protein levels by western blotting. Figure 3.17(a) shows that the levels of both ATM and ATR decreased in the shcontrol cells treated with cisplatin, suggesting that they are not responsible for the S-phase arrest observed in these cells. This result was corroborated by the observation that there was a corresponding reduction in levels of the ATR effector kinase chk1. Interestingly, these pathways seem to be activated in the shTBX2 cells since the levels of ATM and chk1 increased and the ATR protein was present at high levels. It is worth mentioning that the levels of the phosphorylated forms of ATM, ATR and chk1 would be the best indicators of their activities, however the reduced levels of total protein observed in the shcontrol cells suggests that the above proteins are not activated under these conditions. These results suggested that knocking down TBX2 may lead to the activation of the ATM and ATR pathways but the significance of this is discussed in chapter 4.

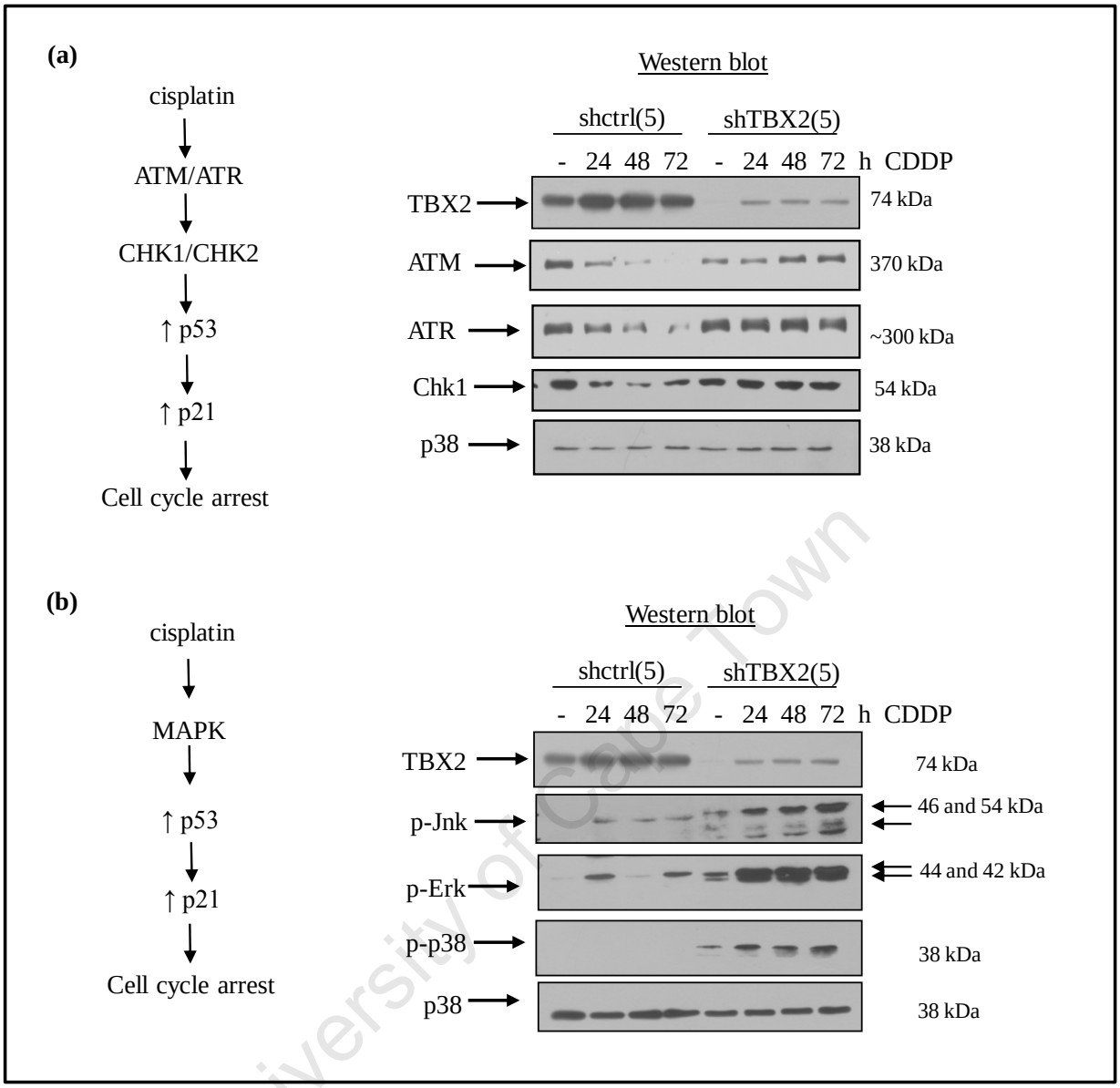

Figure 3.17. Knocking down TBX2 affects both the ATM/ATR and MAPK response. (a) and (b) MCF-7 shcontrol (shctrl) and shTBX2 cells were treated with 10μM cisplatin (CDDP), total protein was harvested at 24, 48 and 72 h and analysed by western blotting. (a) ATR/ATM DNA damage response and (b) MAPK activation using antibodies to the indicated proteins. p38 was used as a loading control.

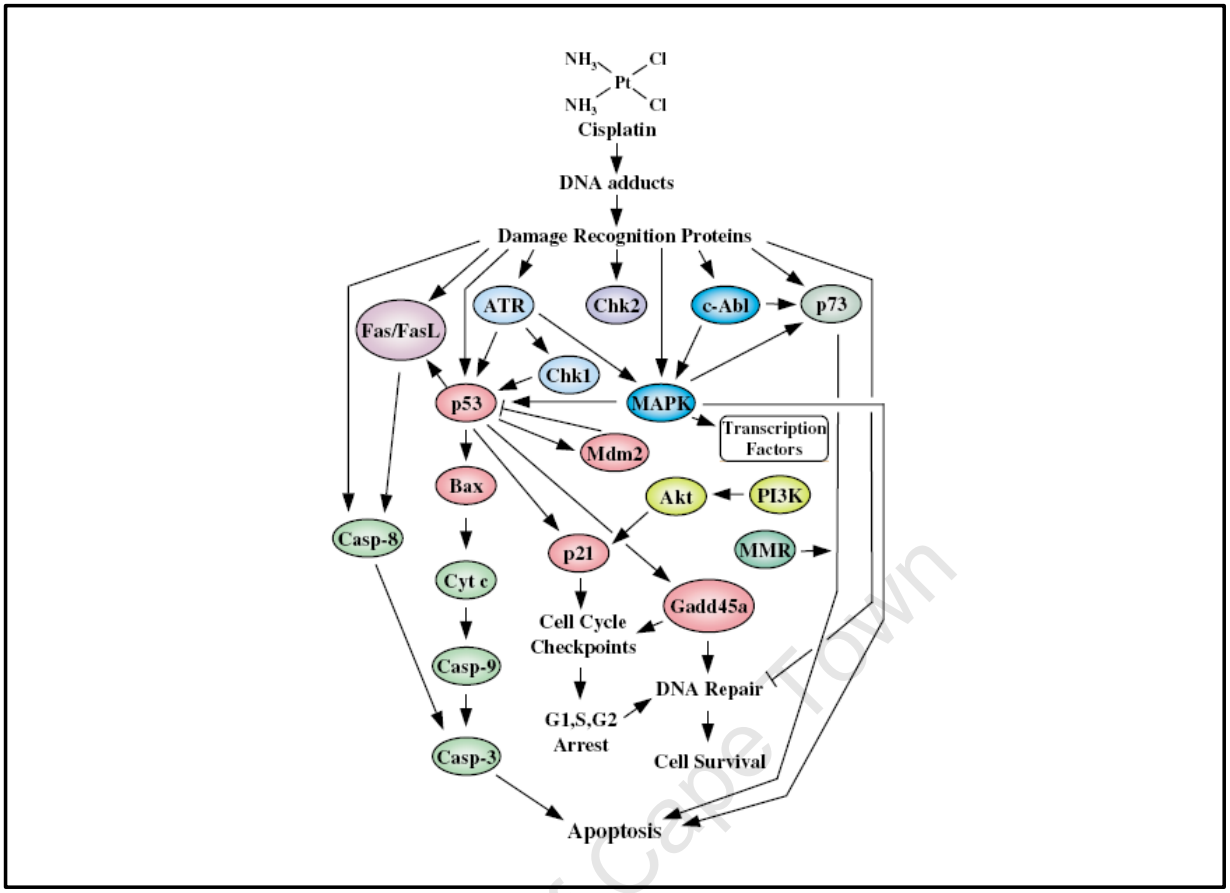

Figure 3.18. A summary of the pathways activated upon cisplatin-induced DNA damage. Depending on the intensity of the signals generated, repair or apoptotic pathways can be activated. Repair pathways can be initiated by a number of proteins including ATR, the MAPKs and p53 (from Siddik, 2003).

Having established that the ATM and ATR pathways did not appear to be involved in the cisplatin response in the shcontrol cells, the role of the mitogen-activated protein kinase (MAPK) pathway was next explored. Figure 3.17(b) shows that both p-Jnk and p-Erk levels increased in the shcontrol cells at all time points of cisplatin treatment. The shTBX2 cells also showed an increase in p-Jnk and p-Erk levels but this response was far more robust at all time points tested. Furthermore, p-p38 levels also increased in the cisplatin treated shTBX2 cells. It is interesting to note that all the MAP kinases tested were not detected in untreated shcontrol cells but they were present in untreated shTBX2 cells. These results suggest that, at the very least, the MAPK pathway is activated in both shcontrol and shTBX2 cells treated with cisplatin which negated their possible role in the S-phase arrest observed in the shcontrol cells.

Considering that all the classical pathways reported to be involved in the cisplatin-induced damage response appear to be intact in the shTBX2 cells, the inability of these cells to sustain an S-phase arrest may be due to TBX2 regulating a protein downstream of the above factors. The tumour suppressor p53 is stabilised and activated by all of the kinases discussed above. Furthermore, the MCF-7 cells are reported to express wild type p53 (Fan et al., 1995; Smith, 1999) and thus the next obvious question that arose was whether the p53 response was intact in the shcontrol cells but impaired in the shTBX2 cells. Western blot analyses showed that the MCF-7 shcontrol cells exhibited a robust increase in p53 levels at 24 and 48 h of cisplatin treatment which was maintained, although at lower levels, at 72 h (Fig. 3.19). Quite excitingly, the MCF-7 shTBX2 cells showed undetectable or very low levels of p53 protein during the time course investigated. This provided a compelling explanation for the impaired S-phase arrest observed when TBX2 is depleted in the cisplatin-treated shTBX2 cells.

The negative cell cycle regulators, *p14*^{ARF} and *p21*, are both p53 target genes and they play an important role in initiating cell cycle arrests following DNA damage (El-Deiry et al., 1993). It was therefore hypothesised that the activation of p53 in cisplatin treated shcontrol cells may lead to the upregulation of these proteins. To investigate this possibility the levels of p14^{ARF} and p21 were investigated in both the shcontrol and shTBX2 cells in response to cisplatin treatment by western blotting. Figure 3.19 shows that while there was a dramatic lack of induction of p14^{ARF} in shcontrol cells treated with cisplatin p21 levels increased.

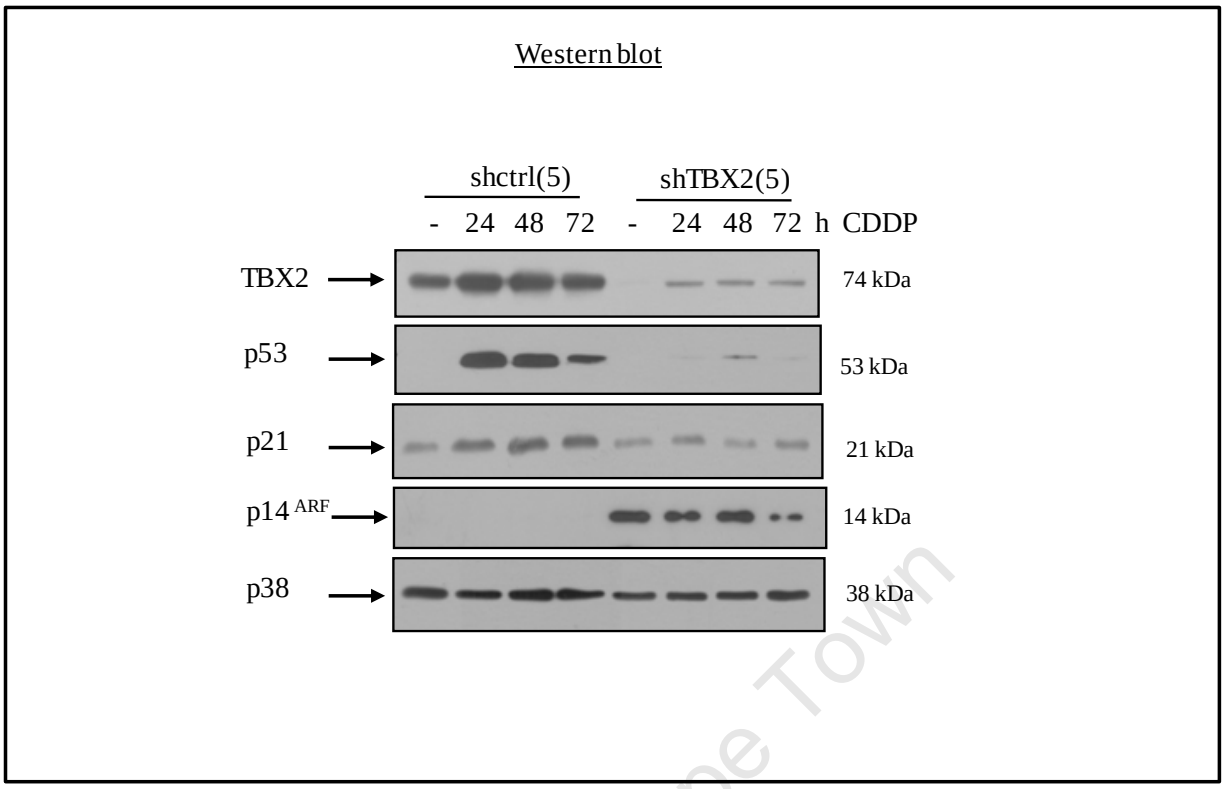

Figure 3.19. TBX2 is required for the induction of p53 in MCF-7 cells following cisplatin treatment. MCF-7 shcontrol (shctrl) and shTBX2 cells were treated with 10 µM cisplatin (CDDP) and total protein harvested after 24, 48 and 72 h. Protein extracts (40 µg) were analysed by SDS-PAGE (8-15%) and western blotting using antibodies to the indicated proteins. p38 was used as a loading control.

These results implicated p21, and not $p14^{ARF}$, in the S-phase arrest observed in cisplatin treated shcontrol cells. This possibility is confirmed by the observation that p21 levels were not upregulated in cisplatin treated shTBX2 cells and explains why these cells did not arrest in S-phase. Results for $p14^{ARF}$ levels in the shTBX2 were particularly interesting for the following reasons. Firstly, in the untreated shTBX2 cells $p14^{ARF}$ levels were particularly high compared to the shcontrol cells suggesting a mechanism for their reduced proliferative ability reported earlier in this study. Secondly, the continued high levels of $p14^{ARF}$ in cisplatin treated shTBX2 cells may be responsible for the apoptosis observed in these cells. This is in keeping with previous reports that $p14^{ARF}$ has a role in mediating p53-independent apoptosis (Fatyol and Szalay, 2001; Hemmati et al., 2002). As mentioned earlier, TBX2 has previously been shown to directly repress $p14^{ARF}$ and *p21* (Jacobs et al., 2000; Lingbeek et al., 2002; Prince et al., 2004). It is therefore possible that silencing TBX2 leads to the derepression of $p14^{ARF}$ in shTBX2 cells. Unpublished data generated in our laboratory however suggest that the p21 response in both the shcontrol and shTBX2 cells may be as a result of a more complex regulation of *p21* by p53 and TBX2 and this will be discussed in chapter 4.

The effect of cisplatin treatment on cell survival when TBX2 is either knocked down or over-expressed (see 3.1.5) suggests that TBX2 may enhance chemotherapeutic drug resistance in TBX2-driven cancers. This makes TBX2 an attractive novel target in the development of anti-cancer drugs.

Chapter 4 Discussion

Several studies have implicated the T-box factor, TBX2, in tumourigenesis. For example TBX2 can prevent senescence in mouse embryonic fibroblasts and ST.Hdh^{Q111} striatal cells through a mechanism involving its ability to repress the negative cell cycle regulators *p19^{ARF}* and *p21* (Jacobs et al., 2000; Lingbeek et al., 2002; Prince et al., 2004). In addition, *TBX2* has been shown to be amplified and/or over-expressed in a subset of breast and pancreatic cancer cell lines and tumours (Erson et al., 2001; Sinclair et al., 2002; Packham and Brook, 2003; Mahlamaki et al., 2002; Chen et al., 2008a; Duo et al., 2009). Furthermore, *TBX2* is over-expressed in a subset of primary melanomas and melanoma cell lines, where it was shown to function as an anti-senescence factor (Wang et al., 2004a; Vance et al., 2005). Although these studies suggest that TBX2 may contribute to the oncogenic process, whether TBX2 plays a causative role in this process and whether this exclusively involves its anti-senescence function has not yet been elucidated. This study addresses these questions through the use of both TBX2 over-expression and knockdown cell culture models. The results show that TBX2 does indeed impact directly on the oncogenic process and further reveals a novel mechanism by which it contributes to tumourigenesis. Importantly, knocking down TBX2 was shown to be sufficient to reduce several features of transformation in both a breast cancer and melanoma cell line that over-express this protein. Furthermore, TBX2 is demonstrated to be associated with cisplatin resistance and knocking down TBX2 sensitises breast cancer cells to cisplatin.

4.1 *Over-expression of TBX2 in transformed human lung fibroblasts*

Genomic instability is thought to promote tumourigenesis because it leads to the acquisition of mutations that favours the transformed phenotype (Nigg, 2002). This study demonstrates that re-expression of TBX2 in a transformed lung fibroblast cell line results in cells that exhibit an increase in frequency of several features of genetically unstable cells. These included chromosomal rearrangements as reflected by derivative and unidentifiable chromosomes and small marker chromosomes. Importantly, giant marker and dicentric chromosomes were present in only the TBX2-expressing cells. Interestingly, small marker chromosomes have been shown to be useful to cancer cells because they can harbour extra copies of genes that encode proteins conferring resistance to chemotherapeutic drugs

(Gisselsson, 2008). The increased number of these chromosomes in CT-TBX2 cells may explain why, although grossly abnormal, these cells are still capable of dividing and give rise to cells that are resistant to the chemotherapeutic drug cisplatin. Furthermore, the reduced proliferation rate of these TBX2 over-expressing cells could be due to increased aneuploidy and chromosome missegregation, which has been reported to compromise proliferation rate (Fan et al., 2003; Williams et al., 2008).

The above results suggest that one mechanism by which TBX2 may contribute to tumourigenesis is through inducing genomic instability. Importantly, the CT-TBX2 cells have much lower levels of the TBX2 protein compared to the normal WI-38 lung fibroblasts. The changes in phenotype observed in the CT-TBX2 cells are therefore not due to gross over-expression of TBX2. It is important to note that the cell line used in this study was already transformed, however attempts at over-expressing TBX2 in a normal cell line were unsuccessful. This is in agreement with other studies (Jacobs et al., 2000; Butz et al, 2004) which have found ectopic expression of Tbx2 in several cell lines to be toxic. Whether TBX2 is able to induce the same genetic instability in normal cells is therefore not known, possibly because they have more robust cell cycle checkpoints which would lead to the elimination of tetraploid cells. Indeed, this is the first report characterising the properties of genetically engineered TBX2-expressing cell lines.

Understanding the mechanism by which TBX2 induces increased genomic instability will be an important topic of future work. More specifically how TBX2 causes the doubling of chromosomes, resulting in polyploidy, in already transformed lines may provide a clue to this mechanism. Based on the current literature, polyploidy can be generated from a wide variety of errors in cell division which go unnoticed by complex surveillance mechanisms (Nigg, 2002; Storchova and Pellman, 2004; Kops, 2008). For example, anaphase chromosome segregation and cytokinesis are tightly co-ordinated processes that have to be completed with high fidelity and if defects in either of these processes are detected, a spindle assembly checkpoint (SAC) and/or tetraploidy checkpoint arrest are imposed. In the event that these surveillance mechanisms are compromised cells can “slip past” this arrest (Storchova and Pellman, 2004) resulting in polyploid cells. Initially, flow cytometry analysis of CT-TBX2 cells showed a predominantly 4N population, indicating that they were experiencing a cell cycle block in G2 or M which prevented them from undergoing cytokinesis. An alternative

possibility as to the identity of the 4N population observed in the CT-TBX2 cells in figure 3.3(b) is that this represents an already polyploid population arrested in G1. In this instance a G1 cell cycle arrest could represent activation of the tetraploidy checkpoint or a senescent arrest. It has been speculated here that the already tetraploid nature of the CT-1 cells may indicate that these cells have a defective tetraploidy checkpoint. It may be possible however that the increased abnormalities observed in the CT-TBX2 cells initiated senescence pathways, but TBX2's ability to bypass senescence resulted in these cells continuing to cycle. This would explain the flattened morphology observed for the CT-TBX2 cells. The presence of lagging chromosomes and anaphase bridges in CT-TBX2 cells, however, supports the possibility that the block is in M phase and therefore possibly due to a mitotic spindle checkpoint arrest. This would therefore suggest that the parental CT-1 cells have a functional SAC. Indeed, flow cytometry analyses of CT-E and CT-TBX2 cells treated with nocodazole showed that these cells were capable of arresting in mitosis, indicative of a functional SAC. However, with increasing passage of CT-TBX2 cells, a subpopulation of cells was able to proceed through the cell cycle undetected with a DNA content of 8N. Altered TBX2 protein levels may therefore, in addition to affecting chromosome segregation, compromise anti-polyploid surveillance mechanisms found downstream of SAC. It is possible that TBX2 may do this through regulating key cell cycle regulators especially since TBX2 has been shown to interact directly with and repress the cell cycle regulators $p19^{ARF}$/ $p14^{ARF}$ and *p21* (Jacobs et al, 2000; Brummelkamp et al, 2002a; Prince et al, 2004). In this study the changes observed in the CT-TBX2 cells were not associated with alterations in p53, p21 or cyclin B1 protein levels but with increased levels of $p14^{ARF}$. The increased $p14^{ARF}$ levels may be responsible for the G2/M arrest and/or the apoptotic sub G1 peak initially observed in the CT-TBX2 cells. Indeed, reports in the literature show that $p14^{ARF}$ can function independently of p53 to initiate both cell cycle arrest and apoptosis (Fatyol and Szalay, 2001; Hemmati et al., 2002). This would suggest that the increase in $p14^{ARF}$ is not due to a direct regulation of the gene by TBX2 but rather due to a cellular response to TBX2 over-expression.

The mechanism(s) by which the over-expression of TBX2 induces mitotic defects may be linked to its levels needing to be tightly regulated during the cell cycle. Indeed, an article by Bilican and Goding (2006) demonstrated that Tbx2 protein levels are regulated during the various stages of the cell cycle with the levels peaking at G2 and being greatly diminished in mitosis. Interestingly, in CT-TBX2 cells, TBX2 protein levels follow the same general

pattern as described by Bilican and Goding (2006), except that it remain high in M-phase. It is therefore tempting to speculate that when TBX2 is over-expressed, as is the case in some cancers, the mechanism by which the protein is degraded after G2 is compromised and hence the protein interferes with mitotic events. This would account for the lagging chromosomes and anaphase bridges seen in CT-TBX2 cells. This possibility is consistent with other cell cycle regulators such as cyclin A and cyclin B1 which are important for G2 and entry into M-phase but their destruction is required for the completion of mitosis. Interestingly, work in our laboratory has shown that TBX2 interacts with cyclin A and cyclin B1 and that the protein is phosphorylated by the cyclin A/CDK2 and cyclinB1/CDK1 complexes (unpublished data). It is therefore possible that TBX2 also needs to be degraded/downregulated for a successful mitosis. The data presented here are therefore consistent with, and provide functional support for, reports identifying a role for TBX2 in G2 or entry into mitosis.

4.2 *Silencing TBX2 expression in MCF-7 breast cancer and 501 melanoma cells*

Evidence that increased levels of TBX2 plays a causative role in the oncogenic process was further demonstrated in this study when silencing TBX2 was shown to abrogate a number of key features of transformation. Results obtained show that knocking down TBX2 in both the MCF-7 breast cancer and 501 melanoma cell lines significantly reduced the cells ability to proliferate. Furthermore, TBX2 was required by these cancer cells to proliferate independently of mitogenic stimuli and a substrate. Interestingly however, reducing TBX2 levels did not affect the migration rate of either cancer cell line. These data would suggest that TBX2 contributes to the oncogenic process primarily by functioning as a powerful pro-proliferative factor rather than contributing to invasive ability. This is consistent with previous reports that silencing Tbx2 expression using a dominant negative approach induced senescence in B16 mouse melanoma cells and that expression of Tbx2 in *BMI*$^{-/-}$ mouse embryonic fibroblasts enabled these cells to bypass senescence under conditions where control cells senesce (Jacobs et al., 2000; Vance et al., 2005). *p14*^{ARF} and *p21* are TBX2 targets (Jacobs et al., 2000; Brummelkamp et al., 2002a; Prince et al., 2004) and results show that while the untreated MCF-7 shcontrol and shTBX2 cells express comparable levels of p21, the levels of p14^{ARF} were substantially elevated in the shTBX2 cells. It is therefore

possible that knocking down TBX2 de-represses expression of $p14^{ARF}$ and consequently re-institutes functional checkpoints in the shTBX2 cell lines. If this were the case then increased levels of TBX2 would be required for the uncontrolled proliferation of TBX2-associated cancers by repressing $p14^{ARF}$.

The finding that reduced TBX2 levels did not affect the migration rate of cancer cells is interesting in the light of recent data obtained in our laboratory for the closely related family member TBX3. While TBX3 had very little effect on proliferation of breast and melanoma cancer cells it was shown to be required for tumour formation and invasion in nude mice. Since the cancer cell lines tested express high levels of TBX2 and TBX3 it may be hypothesised that whereas TBX2 is important for the enhanced proliferative ability, TBX3 is required for their metastatic potential.

The observation that shTBX2 cells appear to spend a much longer time in S-phase compared to the shcontrol cells deserves some comment. It is well established that replication initiates at discrete foci which can be observed by punctate staining when pulsing with BrdU (Mills et al., 1989). In human cells DNA replication is completed in approximately 9 h and can be visualised as uniform nuclear BrdU staining (Berlingin et al., 1992). The observation that after 36 h of BrdU treatment, 50% of asynchronous shTBX2 cells, compared to 10% shcontrol cells, exhibit a punctate BrdU labelling pattern suggests that they struggle to complete DNA synthesis. In addition to this providing an explanation for the reduced proliferative ability of shTBX2 cells, these results may also implicate TBX2 to play an as yet undefined role in S-phase. This is potentially a very important finding that would need to be followed up in future experiments.

The results from the knockdown experiments prompted the investigation of whether TBX2 was a good target for chemotherapeutic drugs. This was particularly relevant for the MCF-7 cells which have been reported to be resistant to the chemotherapeutic drug cisplatin (Fan et al., 1995; Yde and Issinger, 2006). In the current study the MCF-7 shcontrol cells arrested in S-phase at 24 h of cisplatin treatment and this correlated with a robust increase in p53 levels. It was therefore speculated that the S-phase arrest may enable the control cells to repair the cisplatin-induced damaged and indeed, the undetectable levels of the DNA damage marker γ-H2AX at 72 h of cisplatin treatment support this. This result is consistent with reports in the

literature which show that one mechanism by which cancer cells can develop drug resistance is by triggering cell cycle checkpoints that enable DNA repair and consequently escaping apoptotic pathways (Luqmani, 2005; Levesque and Eastman, 2007). This strategy was first hypothesised by Lau and Pardee (1982) following the observation that caffeine enhanced the toxicity of the alkylating agent nitrogen mustard 2-chloro-N-(2-chloroethyl)-Nmethylethanamine (HN2) by forcing damaged G2 cells through a lethal mitosis. Caffeine is an inhibitor of ATM and ATR, and so leads to ineffective S and G2-phase DNA damage response checkpoints (Sarkaria et al., 1999). Indeed, the resulting cells displayed nuclear fragmentation, indicative of the cells undergoing mitotic catastrophe (Castedo et al., 2004). In the current study knocking down TBX2 resulted in cells which had barely detectable levels of p53, were unable to arrest in S-phase and were sensitive to cisplatin treatment as demonstrated by the presence of markers of mitotic catastrophe. It is therefore tempting to speculate that this abrogated S-phase arrest is what resulted in these cells progressing through the cell cycle with damaged DNA and undergoing apoptosis. The finding that knocking down TBX2 sensitised the MCF-7 breast cancer cells to cisplatin was particularly exciting because it suggests that TBX2 would be a good target for anti-cancer drugs. Furthermore, these results raise the possibility that one of the consequences of TBX2 over-expression in cancers is increased cisplatin resistance which is supported by the results obtained for the CT-TBX2 cells in this study.

The mechanism by which TBX2 confers cisplatin resistance to MCF-7 cells in this study appears to be through a p53-dependent S-phase arrest which allows DNA damage repair. This involvement of p53 in conferring cisplatin resistance is consistent with several other reports in the literature. For example, Yazlovitskaya et al. (2001) demonstrated that cisplatin resistance was associated with prolonged stabilisation and accumulation of wild type p53 in ovarian carcinoma cells. Likewise, head and neck squamous cell carcinoma cell lines which were originally cisplatin sensitive became resistant to this drug as a result of gain of p53 function (Bauer et al., 2005). In addition, whereas human cells harbouring wild type p53 were able to successfully remove cisplatin-induced DNA cross-links, those with mutant p53 were unable to do so (Bhana et al., 2008). This suggests that the decision to activate repair or apoptotic pathways is critical in determining whether or not a cell develops cisplatin resistance. It is therefore not surprising that a current line of thought is emerging that effective chemotherapeutic drugs would need to target factors that enable cells to repair their

DNA, thus forcing cells into a mitotic catastrophe. In this strategy TBX2 would become an important target.

The current study demonstrated that TBX2 was required for a robust p53 induction but due to time constraints the mechanism by which this occurs could not be followed up. While there is currently no evidence that TBX2 can transactivate a physiologically relevant target, a weak activation domain has been identified in the N-terminal of the protein (Paxton et al., 2002). Our laboratory is thus presently exploring the possibility that p53 is transcriptionally activated by TBX2. Interestingly, co-immnoprecipitation experiments performed in our laboratory have shown a direct interaction between the TBX2 and p53 proteins which may suggest that TBX2 is required for an increase in p53 protein stability. Furthermore luciferase reporter assays show that the ratio of TBX2 to p53 protein levels is important in the regulation of the *p21* promoter by p53. These results beg the question as to why TBX2 would promote the activation of *p21* in some instances and directly repress it in other instances. The answer to this is not known but the above results allude to a complicated regulatory mechanism.

The ATM, ATR and MAP kinase signalling pathways have been shown to be activated in response to cisplatin in a number of different cell lines (reviewed by Siddik, 2003). The results from this study show that, among the pathways tested, the Erk and Jnk MAP kinases appear to be the only pathways involved in the cisplatin response in the MCF-7 control cells. While it is established that Erk plays a role in the cisplatin-response in MCF-7 cells (Kim et al., 2005; Eckstein et al., 2008), the current study appears to be the first to link Jnk to the cisplatin response in these cells. Compared to the shcontrol cells, the shTBX2 cells expressed elevated levels of ATM and ATR and showed a dramatic increase in the activation of all three MAP kinases analysed when treated with cisplatin. It is important to note that in response to cellular stress, the activation of ATM, ATR and MAPK signalling pathways leads to inhibition of cellular proliferation and/or decreased cell survival (Robinson and Cobb, 1997; Abraham, 2001). Indeed, Kim et al. (2005) showed that when the Erk response was enhanced in MCF-7 cells this resulted in increased cisplatin sensitivity and studies in human Jurkat leukemic T-cells have shown that cisplatin induced apoptosis is dependent on prolonged Jnk activation (Krilleke et al., 2003). Therefore the enhanced activation of the MAPK pathways and increased levels of ATM and ATR in the shTBX2 cells may be as a

response of activated apoptotic pathways. Furthermore, the observation that the levels of all MAPKs tested were elevated even in the untreated shTBX2 cells suggest that knocking down TBX2 may be causing some kind of cellular stress, possibly due to the reduced replication rate, but whether this affects the pathways activated in the cisplatin response is not known.

In conclusion, the results obtained from this study undoubtedly show that TBX2 does indeed play a causative role in the oncogenic process. This was illustrated by the significantly reduced transforming potential when TBX2 was knocked down in both a breast cancer and melanoma cell line which over-express TBX2. Furthermore over-expression of TBX2 in transformed fibroblasts increased the frequency of several features of genomic instability such as chromosome missegregation, chromosomal rearrangements and polyploidy. These results reveal a novel mechanism by which TBX2 may contribute to the oncogenic process. Finally, results from both TBX2 knockdown and over-expression studies with cisplatin treatment suggest that TBX2 may enhance chemotherapeutic drug resistance in TBX2-associated cancers. This makes TBX2 an attractive novel target in the development of anti-cancer drugs.

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Chapter 6 Appendix

6.1 *Generation of pSuper.neo/GFP siRNA constructs*

Oligonucleotide annealing buffer
100 mM Potassium Acetate
30 mM Hepes Buffer pH7.4
2 mM Magnesium Acetate

6.2 *Cell culture*

Mycoplasma test mounting fluid
20 mM Citric acid
55 mM $Na_2HPO_4.2H_2O$
50% Glycerol
pH to 5.5 and store at 4°C

6.3 *Growth curves*

BrdU incorporation
Carnoys fixative:
1:3 acetic acid:methanol

Borate buffer (0.1 M):
3.8 g sodium borate (borax)
Make up to 100 ml, pH to 8.5

6.4 *Immunofluorescence*

Paraformaldehyde (4%)
4 g paraformaldehyde
Dissolve in 100 ml PBS at 60°C, allow to cool and store at -20°C

6.5 *Protein harvest and western blotting*

RIPA
150 mM NaCl
1% Triton X-100
0.1% SDS
20 mM Tris (pH 7.5)
1% deoxycholate
Protease inhibitors added prior to harvesting: 1X complete protease inhibitor tablets (Roche, Germany), aprotinin (1 µg/ml), pepstatin (1 µg/ml), phenylmethanesulphonyl fluoride (PMSF) (0.5 mM)

Sodium Dodecyl Sulphate (SDS)-polyacrylamide gels

Resolving gel:

Acryl-bisacryl-amide mix (30:08) (percentage depending on size of protein of interest)
0.375 M Tris (pH 8.8)
0.1% SDS
0.1% TEMED
0.1% Ammonium persulphate

Stacking gel:

5% Acryl-bisacryl-amide mix (30:08)
0.192 M Tris (pH6.8)
0.1% SDS
0.1% TEMED
0.1% Ammonium persulphate

Acryl-bisacryl-amide mix (30:08):

29 g acrylamide
1 g N,N`-methylenebisacrylamide
Make up to 100 ml, heating at 37°C to dissolve chemicals. Store at 4°C, protected from light

Running buffer:

1 g SDS
3.03 g Tris
14.41 g glycin
Make up to 1 litre

Transfer buffer:

2.9 g glycine
5.8 g Tris
0.37 g SDS
200 ml isopropanol
Make up to 1 litre and store at 4°C.

Phosphate buffered saline (PBS)/Tween

10X PBS:

8 g NaCl
1.45 g $Na_2HPO_4.12H_2O$
0.2 g KCl
0.2 g KH_2PO_4
Make up to 1 litre, pH to 7.4.

PBS/Tween:

For membrane washes, add 0.1% Tween to 1X PBS

Stripping buffer

62.5 mM Tris-HCl (pH6.7)

2% SDS
100 mM β-mercaptoethanol

6.6 *Flow cytometry*

Propidium iodide solution

2 mM $MgCl_2$
10 mM Pipes buffer
0.1 M NaCl
0.1% Triton X-100
0.01 mg/ml Propidium iodide

6.7 *pSuper.neo/GFP vector map*

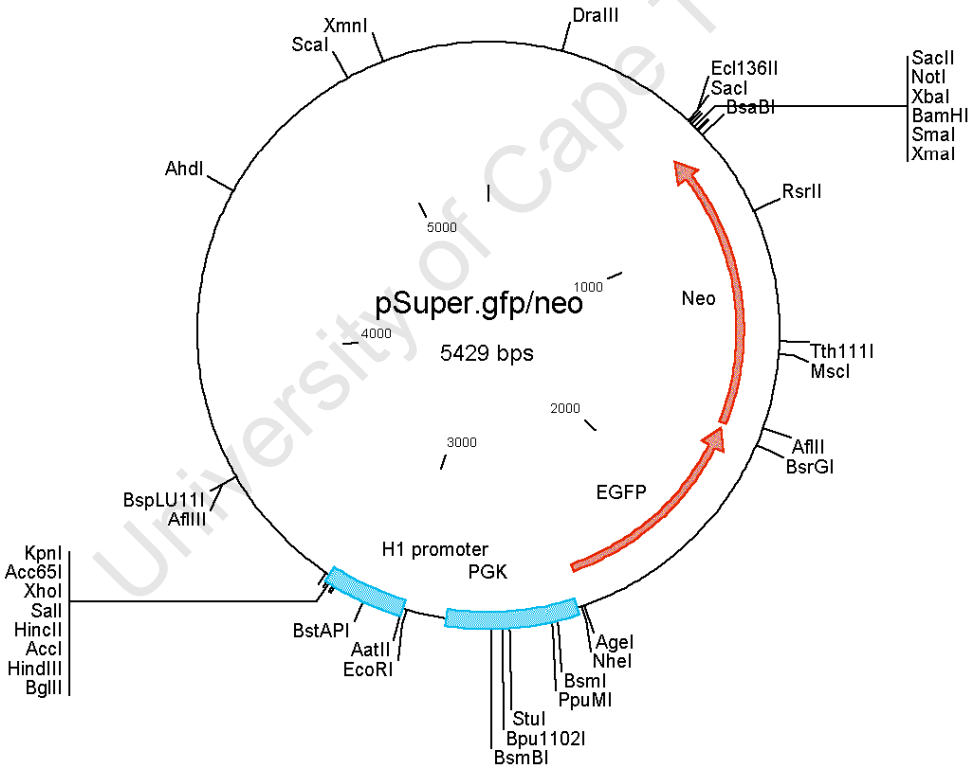

Figure 5.1. pSuper.neo/GFP (OligoEngine, Seattle, USA) vector map. The RNA transcript, leading to the synthesis of siRNA-like molecules is driven off the promoter of the H1 RNA polymerase III gene. In this instance, both the TBX2 and control sequences were cloned into the multiple cloning site as *Bgl*ll *Hind*lll fragments. A PGK promoter drives the expression of various selective marker genes, including EGFP to monitor maintenance of the plasmid, and ampicillin and neomycin resistance genes as selective markers in bacterial and cell culture systems respectively.