

**E2F1-mediated transcriptional regulation**

*in vitro and in vivo*

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## **1. Abbreviations**

ATM	ataxia-telangiectasia mutated
Apaf-1	apoptosis protease-activating factor-1
BCNU	Carmustine
BSA	bovine serum albumin
bp	base pair
Cdk-4	cyclin-dependent kinase-4
Cip	CDK inhibitor protein
CNS	central nervous system
DP	dimerization protein
Da	Dalton
DHFR	dihydrofolate reductase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine- tetraacetic acid
EtOH	Ethanol
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
GBM	Glioblastoma multiforme
GFP	Green fluorescent protein
IgG	Immunoglobulin G
INK4	inhibitor CDK4
IP	immunoprecipitation
IRES	internal ribosome entry site
kDa	Kilodalton
LTR	long terminal repeat
LUC	luciferase

M	Molar
MRI	Magnetic resonance imaging
MDM2	murine double minute clone 2
MW	molecular weight
min	minute
mRNA	messenger-RNA
NLS	nuclear localization signal
nM	nanoMolar
nt	nucleotide
N-terminus	Amino-terminus
O.D	Optical density
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
RNA	Ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate
siRNA	small-interfering RNA
ser	serine amino acid
TK	thymidine kinase
Ub	ubiquitin
wt	wild-type
μ	micro
WHO	world health organization

## **2. Introduction**

Of all cancer types, brain tumours probably represent the most devastating and difficult-to-treat cancer. Although enormous advances in treating other solid cancers, such as lung and breast cancers, highlighted the last decade, median survival for glioblastoma multiforme (GBM; the most common and aggressive primary brain tumour) stayed nearly the same over the last 50 years, averaging ~ 1 year (Hoffman et al., 2006; Thuppal et al., 2006). Regardless of advances in surgical and imaging techniques, we still face multiple problems when treating brain tumours, some because of extensive infiltration of tumour cells, their invasion into normal brain parenchyma or other sites, and resistance to standard radiation and chemotherapy. However, our paramount inability to successfully treat brain cancer mostly stems from the lack of understanding of the underlying brain tumour biology. Another problem common to diagnosing and thus treating the disease results from difficulties in classifying brain tumours often defined by histological rather than molecular criteria. For example, histological similar primary GBMs arising de novo and secondary GBMs progressing from low- to high-grade tumours will be classified and treated the same regardless of the vast disparity in molecular characteristic (DeAngelis, 2001; Kleihues & Ohgaki, 1999) . Although curing brain cancer might be in the distant future, we have gained some understanding of genetic and molecular characteristics of human brain tumours (Louis et al., 2001). Recent advances in laboratory techniques and successful development of brain tumour models faithfully recapitulating the human disease shed further light on necessity and sufficiency of genetic alterations for brain tumour initiation, progression, and maintenance; help identify relevant therapeutic targets; and provide an amenable field for testing novel therapeutic agents, translating into a better treatment for brain tumour patients.

## **2.1 Brain tumours**

### **2.1.1 Classification and histology**

Normal brain is composed of a variety of cell types, including neurons, glia (astrocytes, oligodendrocytes, microglia, and ependymal cells), vascular epithelium, and meningeal cells. Primary brain tumours can be classified into gliomas, the most common adult brain tumours occurring in the brain parenchyma above the tentorium, and medulloblastomas, child or young adult cerebellar tumours occurring below the tentorium (Lassman, 2004). Intracranial tumours arising from meninges (meningiomas) and tumour metastases from systemic cancers (lung, breast, colon) are not considered primary brain tumours and will not be discussed.

Depending on morphologic and histologic similarities to the normal cells, gliomas can be classified as astrocytic, oligodendrocytic, or mixed, and are proposed to arise from the respective cell type (Kleihues & Sobin, 2000; Lassman, 2004). The WHO classification divides astrocytic tumours into grades 1 to 4, grade 4 GBM being the most aggressive and malignant primary glioma. Astrocytic tumours (including GBMs) are composed of glial fibrillary acidic protein (GFAP)-expressing cells with fibrillary cytoplasm and angular nuclei, whereas GFAP-negative oligodendroglial tumours present uniform cells with rounded nuclei. GBM features include microvascular proliferation, nuclear atypia, presence of giant cells, and pseudopalisading necrosis. Histologically indistinguishable but molecularly/ genetically distinct primary and secondary GBMs develop as primary *de novo* tumours or progress from the diffuse grade 2 to anaplastic grade 3 astrocytomas and to secondary GBMs. Similar WHO classification subdivides oligodendrogliomas into grade 2 low-grade and grade 3 anaplastic oligodendrogliomas. Although patients with low-grade gliomas survive for 10 to 15 years, patients with grade 3

astrocytomas survive for only 2 to 3 years; the mean survival for patients with a GBM is ~ 1 year (DeAngelis, 2001; Kleihues & Sobin, 2000).

Medulloblastomas, “blue-cell tumours” composed of small cells with little cytoplasm, are locally invasive and express synaptophysin and NeuN. These cerebellar tumours are classified as desmoplastic, if they contain large-cytoplasm cell islands in the field of more typical medulloblastomas cells, or as large cell, if they contain large pleiomorphic cells. Medulloblastomas show good prognosis with a 65% to 85% cure rate and a high possibility of complete resection; yet, CSF tumour cell spreading and severe neuroaxis irradiation effects still pose a problem (Kleihues & Sobin, 2000; Lassman, 2004).

### **2.1.2 Etiology**

The etiology of malignant gliomas is largely unknown. However, there are some hereditary tumour syndromes associated with primary brain tumours such as neurofibromatoses 1 and 2, Li-Fraumeni syndrome and Turcot’s syndrome (Louis & von Deimling, 1995). Also several occupations, environmental carcinogens, and dietary factors have been reported to be associated with an elevated glioma risk, but this data is controversial. Exposure to therapeutic ionizing radiation has been associated with an increased brain tumour risk (Nygaard et al., 1991; Ohgaki & Kleihues, 2005).

### **2.1.3 Genetic abnormalities**

Neoplastic transformation in the central nervous system is a process in which the normal control of the cell proliferation and cell-cell interactions are lost and a normal cell is transformed into a premalignant cell. This process

has been found to be involved in oncogenes, tumour suppressor genes, DNA repair genes, and cell death genes. The alterations of these genes are not specific in malignant glioma but the combination and accumulation are characteristic features (Aguzzi et al., 1995; Maher et al., 2001).

In low grade astrocytomas, overexpression of platelet-derived growth factor (PDGF) ligands and receptors is the earliest alteration in the genome suggesting that autocrine stimulation, cellular proliferation and migration are involved (Guha et al., 1995; Maher et al., 2001). PDGF-receptor overexpression is closely correlated with the mutations of tumour suppressor gene p53 on chromosome 17p (Hesselager et al., 2003). p53 alterations are present in nearly 50% of gr II astrocytomas (Stander et al., 2004). The p53 gene has an important role in cell cycle arrest, response to DNA damage, apoptosis, angiogenesis, and differentiation. Many other growth factors and their receptors are overexpressed in astrocytomas including epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) leading to microvascular proliferation, as well as an increased tendency to infiltrate and migrate (Maher et al., 2001; Plate et al., 1992). Epidermal growth factor receptor (EGFR) gene is overexpressed or its receptor is amplified in 50-60% of all GBM especially in primary GBMs but in few AAS (Marquez et al., 2004; Suzuki et al., 2004). These changes in EGFR facilitate mitogenesis and inhibit apoptosis in tumour cells (Nagane et al., 1996). In secondary high grade astrocytomas molecular abnormalities have been observed in cell-cycle regulatory complex, which includes the p16, cyclin-dependent kinase-4 (cdk-4), cdk-6, cyclin D1, and retinoblastoma (RB) proteins. Deletions of the p16/cdkn2A gene, amplification of cdk-4, and mutations of RB gene lead to an uncontrolled progression of the cell cycle from the G1 to the S phase (Maher et al., 2001; Nakamura et al., 2001). This pathway is altered in 60% of anaplastic astrocytomas and in the majority of GBM (Ichimura et al., 1996).

Loss of heterozygosity (LOH) on chromosome 10q is found in over 60% of GBM (Ohgaki et al., 2004). The tumour suppressor locus is present in chromosome 10 and alterations of PTEN/MMAC-1/TEP-1 gene are found in 30% of GBMs (Fiano et al., 2004). The alterations in the PTEN gene may regulate cell migration, but there may be other tumour suppressor genes in the nearby region in chromosome 10 (Karlsson et al., 1993). The most characteristic genetics alteration is LOH 1p and 19q in oligodendrogliomas, which is evident in 80% of these tumour types. The anaplastic oligodendroglioma has usually a similar progression in the associated genetic changes as high grade astrocytoma (Reifenberger et al., 1994). Some oligoastrocytomas carry tumour suppressor gene p53 mutations related to astrocytoma and some have 1p and 19q deletions related to oligodendroglioma (Maintz et al., 1997).

#### **2.1.4 Current therapeutic approaches**

Glioma and medulloblastoma treatments normally include tumours resection, histologic examination, radiation therapy, and chemotherapy. The most common chemotherapeutic agents used for glioma treatment are DNA alkylating cytotoxic drugs such as carmustine [1,3-bis(2-chloroethyl-1-nitrosourea)]; a triple combination of procarbazine, cisplatin, and vincristine; or the recently development temozolomide etoposide, or lomustine, whereas those used for medulloblastoma treatment are cisplatin, cyclophosphamide, or vincristine.

Chemotherapy is a method of treatment where specific chemical compounds are used to interfere with tumour cell growth and survival. These compounds achieve their effectiveness from their ability to alter the cell cycle or kill the cell. Both these rely on the fact that tumour derived cells are actively

dividing, whereas normal brain cells mostly are not. Therefore, non-dividing cells are usually unaffected by chemotherapy. However, the success of the treatment depends on dividing rate of cancer cells and thus, fraction of cells in this phase of the cell cycle.

However, drugs listed above present serious side effects and eventually fail as patients develop resistance, whereas radiation and chemotherapy adversely affect neurocognitive and physical development (Hu & Holland, 2005; Weiss et al., 2003).

### **2.1.5 Animal models and *in vivo* imaging technologies**

*In vivo* modeling provides essential tumour host interactions and is a more accurate means of modeling human cancer. Mouse models can be classified as Xenograft tumour models or models of spontaneous tumour formation in genetically engineered mice (GEM), and help address issues of utmost importance in drug development: toxicity and *in vivo* antitumour effectiveness. Although the Food and Drug Administration does not require the latter before proceeding to clinical trials, *in vivo* modeling of drug efficacy is a gold standard required by a majority of pharmaceutical companies (Benson et al., 2006; Sellers & Fisher, 1999). Pharmacokinetic/pharmacodynamic models based on physiological and anatomic representation of bodily organs and drug biochemistry are likely to supplement *in vivo* modeling during future drug development (Andersen, 1995; Andersen & Krishnan, 1994).

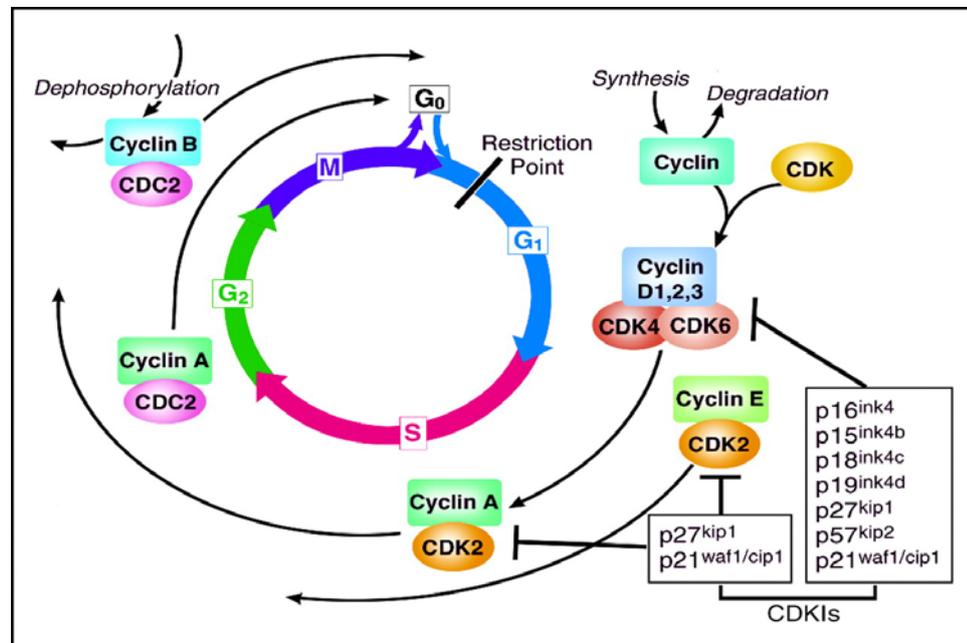
Present imaging tools for living organisms are mostly based on nonspecific physiological or metabolic changes rather than on specific molecular events (Massoud & Gambhir, 2003). Several imaging technologies are available for both humans and animals, including positron emission

computed tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), computed tomography (CT) and ultrasound (US). CT and US are currently used for visualizing anatomical structures such as organs or tumours. MRI has several advantages including high spacial resolution and the ability to gather a broad range of anatomical, molecular and functional information. For molecular imaging, although a reporter probe for a specific target can be used in conjunction with MRI, the sensitivity is relatively low. PET and SPECT generate low spacial resolution images that detect metabolic, molecular and pathological changes with high sensitivity, but both require the use of radioisotope tracers.

Bioluminescence imaging (BLI) and fluorescence imaging are not used clinically, but they have become established methods in animal experiments to monitor grafted tumours (Gross & Piwnica-Worms, 2005; Massoud & Gambhir, 2003). Both of these optical techniques use charge-coupled device detectors to monitor the visible light emitted from the body of anesthetized animals. A reporter gene is engineered to express a protein such as luciferase or green fluorescent protein from a specific promoter. The levels of these proteins can be measured *in vivo* and correlated with the activity of the promoter in the transgene construct. BLI using luciferase has been widely used in cell assay, and is now being applied in living cells and animals. When live animals are provided with luciferin (the luciferase substrate) through injection, luciferase-expressing cells produce light. Fluorescence imaging is visualized using a reporter such as green fluorescence protein and thus requires an excitation light to illuminate the animals, but no injection is needed. Both techniques are highly sensitive and the background noise is minimal for grafted tumours. These reactions are rapid and essentially provide real-time imaging.

## **2.2 Cell cycle overview**

The process of faithfully replicating the genetic information of a cell and its subsequent division into two daughter cells is known as the cell cycle. The cell cycle can be envisioned as alternating cycles of interphase, where the cell grows in size, synthesizes RNA and proteins, and replicates its DNA, and mitosis (M), where the newly replicated cell divides. At times, cells may exit the cell cycle after M and remain quiescent in a resting phase ( $G_0$ ). Once the cell receives a mitogenic stimulus, cells reenter the cell cycle at gap phase 1 ( $G_1$ ), a growth phase prior to replication, followed by entry into synthesis (S), where the DNA of the cell is replicated, succeeded by gap phase 2 ( $G_2$ ), a growth phase preceding cell division in M (Figure 1). Progression through the four stages of the cell cycle is ordered and tightly regulated to ensure that only one round of DNA replication occurs per cell cycle and that cell division only occurs after replication is complete. To combat stressors that cells may encounter as they progress through the cell cycle, such as misaligned chromosomes or damaged DNA, cells elicit checkpoints that halt cell cycle progression and allow the cell to try and amend the problem. However, if the damage is too severe or if repair is untimely, cells will either undergo apoptosis, which is a programmed cell death, or become senescent, which is a state of permanent arrest. Cell cycle arrest and elimination of damaged cells by apoptosis are extremely important processes to an organism in preventing the transmission of damaged or incompletely replicated DNA to new daughter cells and thereby acts as a preventative measure against diseases like cancer.



**Figure 1: The cell cycle**

The cell cycle is divided into four phases (G<sub>1</sub>, S, G<sub>2</sub>, and M) and G<sub>0</sub> resting state. Progression through the cell cycle is promoted by cyclin-dependent kinases (CDKs), which are regulated positively by cyclins and negatively by CDK inhibitors (CDKIs). The restriction point is the point at which cells progress through the cell cycle independent of external stimuli (Schwartz & Shah, 2005).

### **2.2.1 Cell cycle regulation by cyclins and cyclin dependent kinases**

Normal cells need to decide when to divide (ie, enter the cell cycle) and when to stay in  $G_0$ . This is a tightly regulated and carefully balanced process. The entry into the cell cycle ( $G_1$ ) is historically governed by the restriction point- a transition point beyond which cell progression through the cell cycle is independent of external stimuli such as exposure to nutrients or mitogen activation. This point of determination is thought to divide the early and late  $G_1$  phase of the cell cycle.

Progression of a cell through the cell cycle is promoted by a number of cyclin dependent kinases (CDKs). CDKs are a family of serine-threonine protein kinases that control progression of the cell cycle by phosphorylating target proteins at specific times (Morgan, 1995; Pines, 1995). Cdk protein levels remain constant during the cell cycle, so their activity is regulated by expression of the cyclin family of regulatory proteins (Murray, 2004; Pines, 1991; Pines, 1995). Timed expression and rapid ubiquitin mediated proteolysis limits cyclin expression to specific intervals of the cell cycle, limiting the activity of the Cdk to certain phases of the cell cycle (Evans et al., 1983; Glotzer et al., 1991; Murray, 2004; Murray et al., 1989; Reed, 2003). During early  $G_1$ , the cyclin D family (consisting of cyclin D1, D2, and D3) forms a complex with Cdk4 or Cdk6. The cyclin D-Cdk4/6 complex begins the cells entry into S phase primarily by phosphorylation of the retinoblastoma (Rb) family of proteins (Sherr, 1994). This is followed by cyclin E-Cdk2 activity and additional Rb phosphorylation pushing the cell from  $G_1$  into S phase (Ohtsubo et al., 1995). The complex of cyclin A and Cdk2 is required for cells to progress through S phase (Girard et al., 1991; Walker & Maller, 1991). In the  $G_2$  and M phases of the cell cycle cyclin A forms a complex with Cdk1, and this complex is required for transition from  $G_2$  to M (Yam et al., 2002). Mitosis

is then further regulated by activity of the cyclin B-Cdk1 complex (Arellano & Moreno, 1997; King et al., 1994) (Figure 1).

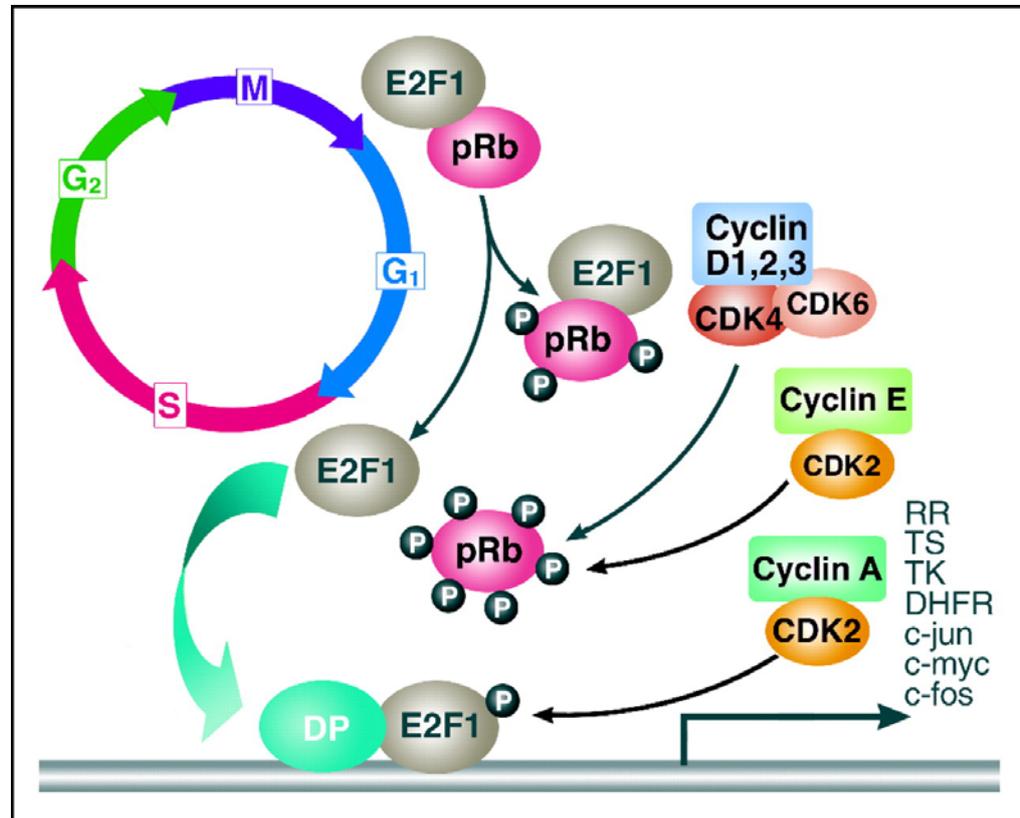
Regulation of these cyclin/cdk complexes is accomplished by a variety of mechanisms (Lee & Yang, 2001; Pei & Xiong, 2005; Pines, 1991; Pines, 1995; Sanchez & Dynlacht, 2005). For example, cyclin/cdk complexes are regulated by a class of proteins known as cyclin dependent kinase inhibitors (CDKi). Cyclin dependent kinase inhibitors play important roles in coordinating proliferation during normal development and differentiation, as well as during cellular stress (Coqueret, 2003; Lee & Yang, 2001). There are two broad classes of CDKi proteins, the INK4 and CIP families (Pei & Xiong, 2005; Pines, 1995; Sherr & Roberts, 1999). The INK4 family, including p16<sup>INK4a</sup> target Cdk4 and Cdk6 and prevent their association with cyclin D (Carnero & Hannon, 1998). Without cyclin D association, Cdk4/6 remain inactive and the cell cycle is unable to progress through G<sub>1</sub> (Quelle et al., 1995; Serrano et al., 1993). The Cip/Kip family of cyclin dependent kinase inhibitors, including p21 and p27, are able to inactivate multiple cyclin-Cdk complexes. They are able to block cell cycle progression in G<sub>1</sub> and, to a lesser extent; S and M phases of the cell cycle and thereby help elicit a checkpoint (Harper et al., 1995; Hengst & Reed, 1998; Lee et al., 1995; Polyak et al., 1994).

### **2.2.2 The Retinoblastoma Family of Proteins**

The Rb protein is a tumour suppressor, which plays a pivotal role in the negative control of the cell cycle and in tumour progression. It has been shown that Rb protein (pRb) governs the G<sub>1</sub>/S transition (Figure 2). In its active state, Rb is hypophosphorylated and forms an inhibitory complex with a group of transcription factors known as E2F-DP (E2F-1, -2, -3), thus controlling the G<sub>1</sub>/S transition. The activity of Rb is modulated by the sequential

phosphorylation by CDK4/6-Cyclin D and CDK2/Cyclin E (Malumbres & Barbacid, 2001; Schwartz & Shah, 2005). When Rb is partially phosphorylated by CDK4/6-CDKs, Rb remains bound to E2F-DP, but this transcription factor is still able to transcribe some genes such as *cyclin E*. Cyclin E then binds to CDK2 and this active complex then completely hyperphosphorylates Rb, thus releasing the E2F-DP complex and fully activating the E2F transcription factors, resulting in transcriptional activation of numerous S- phase proteins, such as thymidylate synthase (TS) and dihydrofolate reductase (DHFR).

The mammalian RB “pocket” protein family consists of three members: Rb, p107, and p130. Each of the RB members has a unique expression pattern during the cell cycle. For example, Rb is present in quiescent cells and then slightly accumulates in cycling cells (Shan et al., 1994). Structurally, there are regions of amino acid similarity between all three RB members, but it is clear that p107 and p130 are more closely related to each other than either protein is related to Rb (Classon & Dyson, 2001).



**Figure 2: Schematic of G<sub>1</sub>-S phase regulation of the eukaryotic cell cycle.**

Hypophosphorylated Rb complexes with the transcription factor E2F. CDK2, CDK4, and CDK6 phosphorylate Rb. E2F is released, binds to DNA with DP, resulting in E2F-dependent transcription for the G<sub>1</sub> to S transition (Schwartz & Shah, 2005).

### **2.2.3 The E2F Family of Transcription Factors**

The E2F transcription factors are key regulators of cell cycle progression and the E2F field has made rapid advances since its advent in 1986. Yet, while our understanding of the roles and functions of the E2F family has made enormous progress, with each discovery new questions arise. Traditionally, the perception of these proteins is that they control the transcription of gene products required for S phase. However, data generated from microarray and chromatin immunoprecipitation analyses have shown that E2F proteins actually regulate a diverse set of genes involved in, not only, cell cycle transitions, but also differentiation, apoptosis, DNA repair, and checkpoint signaling, as well as some unidentified genes (Ishida et al., 2001; Ma et al., 2002; Muller et al., 2001; Polager et al., 2002; Ren et al., 2002).

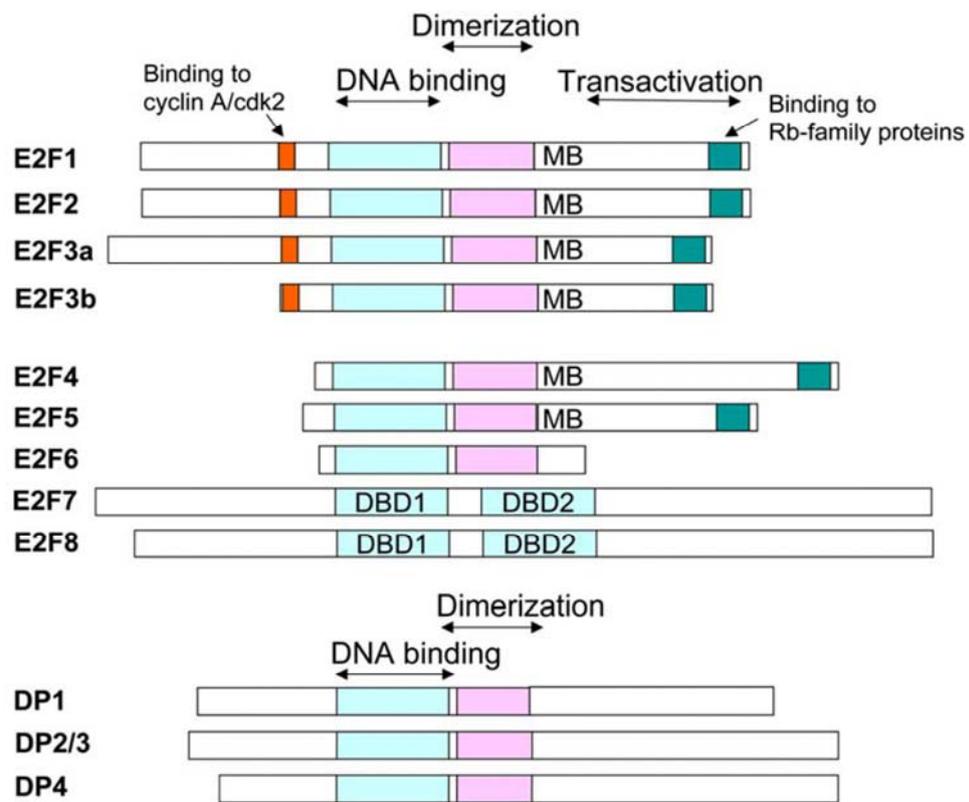
There are now known to be eight E2F family member genes, E2F1-E2F8 (Figure. 3). In addition, two E2F3 proteins, termed E2F3a and E2F3b, are produced from the E2F3 gene through the use of alternative promoters (He et al., 2000; Leone et al., 2000). The highest degree of homology among the E2F family members occurs in the DNA binding domain, which is consistent with the finding that each binds the E2F consensus sequence. Nonetheless, there is accumulating evidence that different E2F family members bind and regulate distinct but overlapping sets of target genes.

E2F1 through E2F6 require dimerization with one of three DP proteins (DP1, DP2/3 or DP4) to form functional transcription factors that bind DNA with high affinity (Bandara et al., 1993; Milton et al., 2006; Ormondroyd et al., 1995; Rogers et al., 1996; Wu et al., 1995; Zhang & Chellappan, 1995). The function of the E2F-DP heterodimer is thought to be determined primarily by the E2F subunit. The roles of the different DP subunits, including several spliced variants of DP2/3 (Ormondroyd et al., 1995; Rogers et al., 1996), in

modulating E2F activity are not completely understood. The transcriptional activity of E2F1 through E2F5 is regulated through their association with RB or the related pocket proteins, p107 and p130. E2F1, E2F2 and E2F3 primarily associate with RB and do so when RB is in the hypophosphorylated state (Lees et al., 1993).

E2F4 and E2F5 can associate with each of the three pocket proteins and the abundance of these complexes changes with the proliferation state of the cells (Gaubatz et al., 2000; Moberg et al., 1996). Pocket protein binding blocks the transcriptional activity of E2F-DP heterodimers by masking the transcriptional activation domains located in the carboxy terminus of E2F1-5.

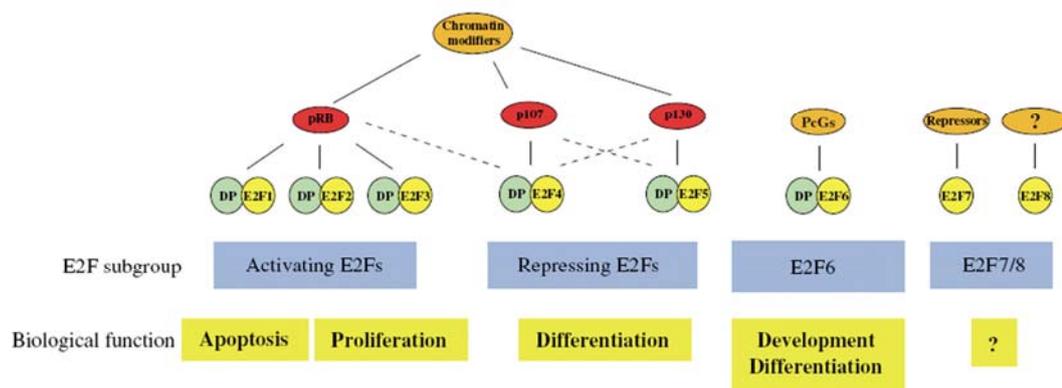
E2F family members have been divided into several subclasses based on their transcriptional regulatory properties on model gene promoters. E2F1, E2F2, and E2F3a are often referred to as “activator” E2Fs because they transcriptionally activate E2F target genes such as *cyclin E*. This subclass of E2Fs is expressed in a cell cycleregulated manner with maximum levels observed in late G<sub>1</sub> and early S phase. Another subclass, which includes E2F3b, E2F4, and E2F5, are referred to as the “repressor” E2Fs because their main function appears to be to repress the transcription of E2F target genes when in association with RB family members (Figure 4). This subclass is expressed constitutively but transcriptional repression by these factors primarily occurs in quiescent and early G<sub>1</sub> phase cells. E2F6, E2F7, and E2F8 also function as transcriptional repressors of E2F target genes but do so independent of RB family members (Cartwright et al., 1998; Christensen et al., 2005; Di Stefano et al., 2003; Logan et al., 2005; Maiti et al., 2005; Morkel et al., 1997)



**Figure 3: Diagrams of the E2F family of transcription factors.**

The highly conserved winged-helix DNA binding domain (DBD) is indicated in blue, and the hydrophobic heptad repeat domain required for dimerization is shown in pink. The DBD also appears to contribute to dimerization. Other domains required for associations with cyclin A/cdk2 and Rb family members are also indicated. E2F1-E2F6 all bind DNA as heterodimers with DP family proteins, while E2F7 and E2F8 appear to associate with DNA independent of DP proteins. Note also that mouse homologs of human DP-2 are usually referred to as DP-3, and there are several differentially spliced isoforms of DP2/DP3 (not shown). There are also two known isoforms of E2F7 which differ in their Ctermini (data not shown) (DeGregori & Johnson, 2006).

It is now becoming clear that this classification of E2Fs as either activators or repressors is overly simplistic and does not accurately reflect the dynamics of E2F-dependent transcriptional control. Microarray analysis has demonstrated that the overexpression of activator E2Fs, such as E2F1, leads to the decreased expression of almost as many genes as are induced (Ma et al., 2002; Muller et al., 2001; Polager et al., 2002). In at least some cases, transcriptional repression by E2F1 is direct and independent of RB (Croxtton et al., 2002; Koziczak et al., 2000; Weinmann et al., 2001). On the other hand, E2F4 and E2F5 associate with several transcriptional co-activators and can transcriptionally stimulate E2F target gene promoters when overexpressed (Beijersbergen et al., 1994; Ginsberg et al., 1994; Lang et al., 2001; Morris et al., 2000). Moreover, recent findings demonstrate that endogenous E2F4 regulates the expression of some genes during the G<sub>1</sub> phase of the cell cycle independent of RB family members (Balciunaite et al., 2005). Chromatin immunoprecipitation (ChIP) assays are revealing surprising complexity in the promoterspecific and context-dependent recruitment of individual E2Fs and E2F-associated co-factors. Some gene promoters conform to the widely accepted model of transcriptional repression correlating with the binding of E2F4/5-p130/p107 complexes in G<sub>0</sub> and early G<sub>1</sub> and then being replaced by E2F1, E2F2, and/or E2F3a in late G<sub>1</sub> and S phase when transcription is induced (Takahashi et al., 2000). However, many other E2F target gene promoters do not fit this model (Balciunaite et al., 2005; Iwanaga et al., 2004; Weinmann et al., 2001; Wells et al., 2003). In summary, several of the E2Fs can either activate or repress transcription through the promoter-specific recruitment of a variety of co-factors and this does not necessarily occur in a cell cycle-regulated manner.



**Figure 4: Schematic representation of the E2F transcription factor subgroups, their physiological roles and specific binding partners:**

The E2F family is divided into at least four subgroups defined by their regulation by pocket proteins and chromatin modifiers, and by their physiological function (i.e. activation or repression). 'DP' presents DP1 or DP2, 'chromatin modifiers' refer to chromatin-modifying activities binding to the pRB family members, such as HDAC. Recent results have shown that also E2F7 recruits repressors to E2F-dependent promoters (Attwooll et al., 2004).

#### **2.2.4 Functional Roles of RB Regulated E2F Activity**

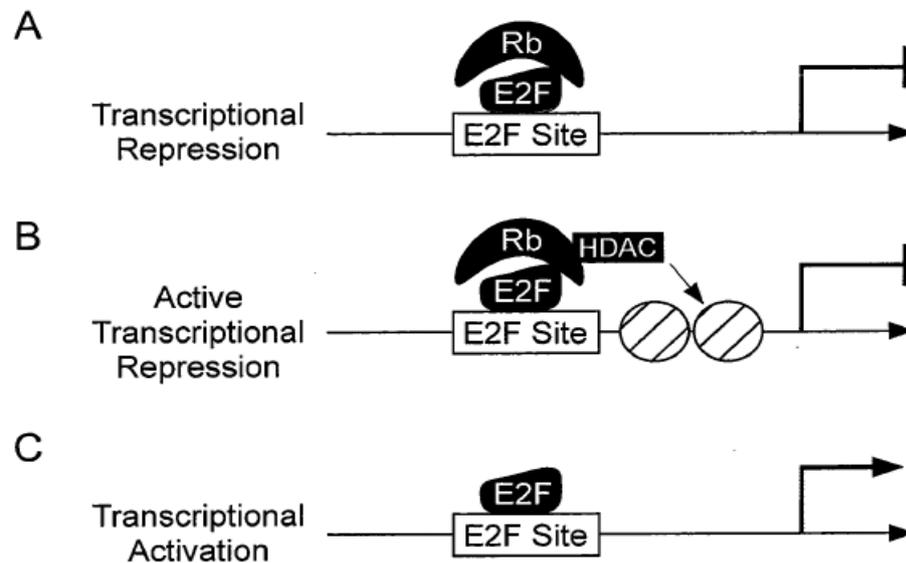
E2F was originally discovered as a cellular binding factor at the promoter of the adenovirus E2 gene (Kovesdi et al., 1986; Kovesdi et al., 1987; Yee et al., 1987). E2F-mediated transcription was stimulated by the adenovirus E1A protein and these events correlated with two forms of E2F, a "free" E2F and a "complexes" E2F (Bagchi et al., 1990). Soon after, Rb was found to be a part of the "complexes" E2F (Bagchi et al., 1991; Bandara & La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991) and E1A was found to bind (Whyte et al., 1988) and inhibit RB (Bagchi et al., 1991; Bandara & La Thangue, 1991; Chellappan et al., 1991). These results led to a model where the complexes E2F was constituted by RB binding and repressing E2F function, and when RB was inactivated, the free E2F was derepressed and able to activate transcription. Although these observations held true for the activator class of E2Fs, the model needed to be rethought once it was observed that E2F/Rb complexes could actively repress transcription (Hamel et al., 1992; Weintraub et al., 1992).

Currently, there are three types of E2F complexes: activator complexes, inhibited complexes (actively repress), and repressor complexes (Figure 5) (Cobrinik, 2005; Dyson, 1998; Nevins, 1998). The activator complexes are E2F/DP heterodimers that actively transactivate promoters. The inhibited complexes are formed when Rb is bound to the activator complex. When Rb binds, it blocks the E2F transactivation domain and makes the complex functionally inert. Finally, active repressor complexes are generated when RB family members are recruited to DNA and actively assemble a repressor activity.

The RB family members mediate repression of E2F function three ways. One way they exert this function is by directly binding and blocking the

transactivation domain of E2F (Flemington et al., 1993; Helin et al., 1993). Additionally, RB is known to bind many proteins involved in chromatin remodeling, such as DNA methyltransferases and histone deacetylases, which actively repress target gene transactivation (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Meloni et al., 1999; Sellers et al., 1995). It has also been observed that RB can physically inhibit pre-initiation complexes from forming at promoters, thereby inhibiting adjacent transcription factor function (Ross et al., 1999).

There are four known mechanisms by which RB can be inactivated. RB inactivation occurs normally during regulated cell cycle progression, where the phosphorylation status of RB and activation of cyclin/cdk complexes is coordinated with the phase of the cell cycle, as described previously (Blagosklonny & Pardee, 2002). The other three mechanisms for RB inactivation result in cell cycle deregulation. One way *Rb* is inactivated is by genetic inactivation, as observed in most cancers (Nevins, 2001; Sellers & Kaelin, 1997; Sherr, 1996; Sherr & McCormick, 2002). Additionally, viral oncoproteins from DNA tumor viruses target the RB proteins (Helt & Galloway, 2003). Finally, Rb is degraded by caspases following apoptotic stimuli (Dou & An, 1998).



**Figure 5: Schematics of the three types of E2F complexes.**

(A) Binding of Rb/E2F complexes to E2F regulated promoters represses E2Fs transactivation function. (B) By recruiting chromatin remodeling enzymes such as HDACs complexes to promoter regions, Rb is able to actively repress transcription. (C) Dissociation of Rb from E2F allows expression from E2F regulated promoters.

### **2.2.5 Cell cycle deregulation and cancer**

Long before the mechanisms of cell cycle deregulation by the small DNA tumor viruses were known, these viruses were found to be associated with tumor formation. Adenovirus was the first human virus identified to induce tumors in an animal model (Trentin et al., 1962). In addition to virus-associated transformation, cancer often results from cellular mutations that result in loss of normal growth control and lead to unrestrained proliferation. The high frequency of cell cycle regulators mutated in human cancers demonstrates the importance of proper cellular growth control for the prevention of cancer (Sherr, 1996). The cell cycle mutations most often observed in human cancers include overexpression of cellular cyclins, loss of cyclin dependent kinase inhibitors, and loss of Rb either by mutation, deletion, or inactivation (McDonald & El-Deiry, 2000).

It is interesting to note that although the overall effect of these mutations is to inactivate Rb and deregulate E2F, mutations in E2F have only recently been found in some rare gastric and colorectal tumors (Iwamoto et al., 2004; Suzuki et al., 1999), suggesting that mutation of E2F might be detrimental to emerging tumor cells.

### **2.2.6 Dual properties of E2F-1: Oncogene and tumor suppressor**

Among the E2F family members, E2F-1 is unique in its ability to regulate a number of key genes that participate in both cell cycle progression and apoptosis, raising a potential link with its role in tumorigenesis. The investigations on cells or animals present us with a wonderful paradox that E2F1 behaves as both oncogene and a tumor suppressor gene. On one hand, enhancement of E2F1 activity in tissue culture cells can stimulate cell

proliferation and be oncogenic. On the other hand, E2F-1 has been demonstrated as a tumour suppressor by spontaneous development of multiple tumours in mice lacking E2F1 (Yamasaki et al., 1996). The biological function of E2F-1 is just beginning to emerge. More recently, new approaches have identified a large number of E2F1 targeted genes involved not only in DNA replication and cell cycle progression, but also in DNA damage repair, check point control, apoptosis (La Thangue, 2003; Stevens & La Thangue, 2003; Trimarchi & Lees, 2002). Thus, E2F-1 is a multi-functional damage responsive protein that is involved in numerous aspects of the DNA damage response (Figure 6).

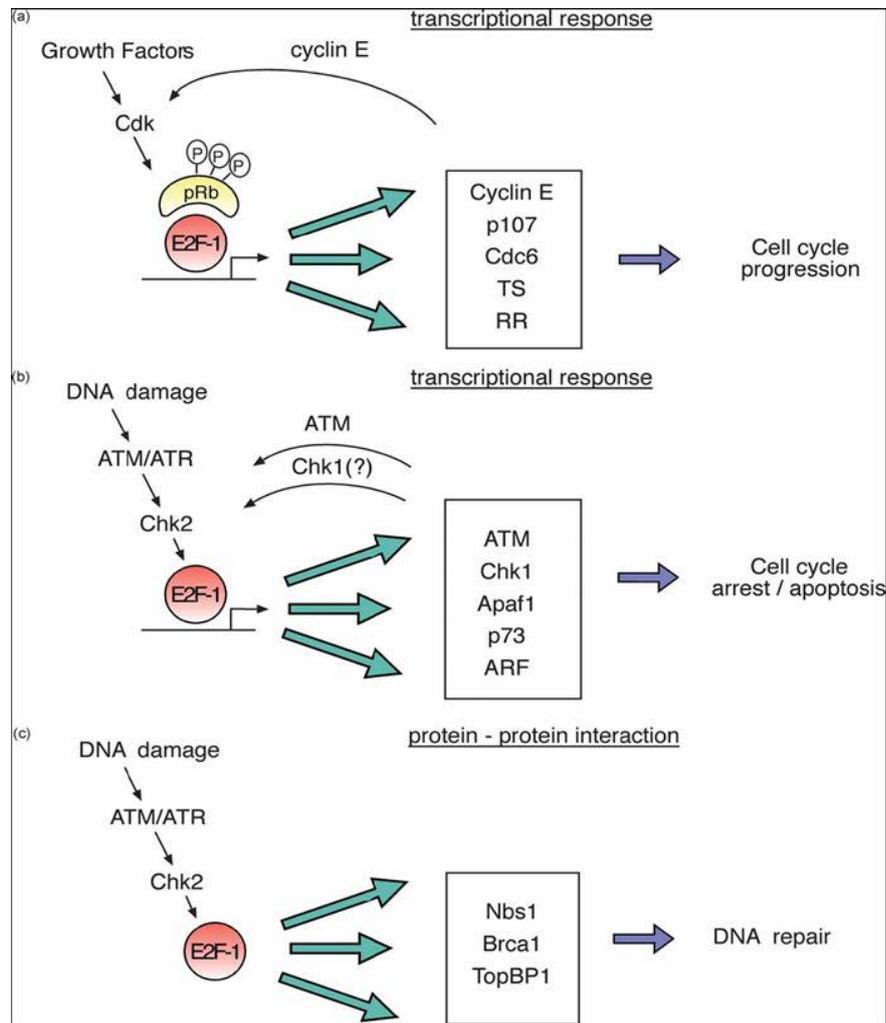
### **2.2.7 DNA damage**

Extracellular and intracellular insults such as reactive oxygen species, ionizing radiation, and radiomimetic drugs can induce DSBs (double-strand breaks) in DNA. Of the many cellular responses to DSBs, regulation of cell division by checkpoints received the most attention initially. Historically, checkpoints refer to mechanisms that arrest the cell division cycle in response to intracellular conditions such as damaged or incompletely replicated DNA. Signals that activate cell cycle checkpoints were later found to activate additional cellular responses such as DNA repair and apoptosis. Thus, currently, these responses, along with cell cycle regulation by checkpoints, are collectively referred to as DNA damage response pathways.

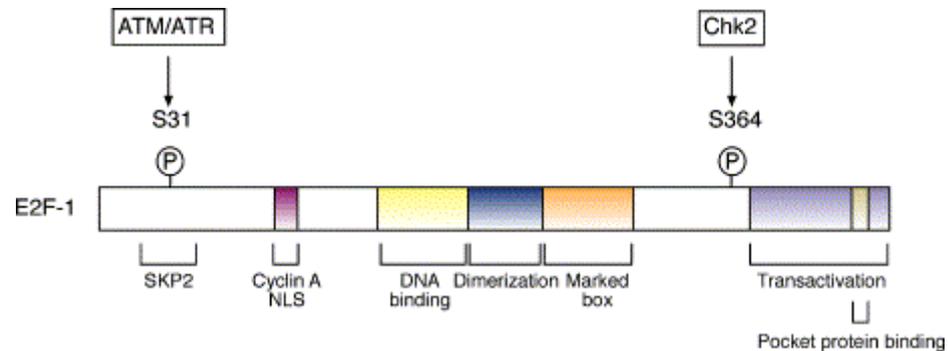
### **2.2.8 E2F1 signaling**

A role for E2F-1 in the DNA damage response pathway was suggested from experiments in which treating cells with DNA damaging agents increased E2F-1 protein levels (Blattner et al., 1999; Hofferer et al., 1999; O'Connor & Lu, 2000). This effect exhibits kinetics that closely resembles the induction of p53. In turn, these results hinted that E2F-1 and p53 may be regulated through a common pathway (Blattner et al., 1999).

We know now that the induction of E2F-1 reflects the fact that E2F-1 is targeted and phosphorylated by ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related (ATM/ATR), and Checkpoint2 (Chk2) protein kinases (Lin et al., 2001; Stevens et al., 2003) ( Figure 7), both of which are integral components of the DNA damage signalling pathway. At present, it is unclear whether ATM/ATR and Chk2 kinases co-operate in regulating E2F-1 activity in response to DNA damage, although it is the case that there is likely to be crosstalk between these phosphorylation events. Whilst we await resolution to the question of the relationship between ATM/ATR and Chk2 in E2F-1 control, it is possible that multiple phosphorylation events ensure that effector proteins are recruited and activated only upon complete activation of the DNA damage response pathways. For example, ATM phosphorylation may be required to prime E2F-1 for subsequent phosphorylation and activation by Chk2 kinase. Alternatively, the level of phosphorylation may modulate different properties of E2F-1; perhaps phosphorylation by ATM is sufficient to signal cell cycle arrest, whereas induction of apoptosis requires phosphorylation by both groups of kinases. The fact that multiple phosphorylation events apply mainly to targets with pleiotropic cellular effects such as p53 lends some weight to this idea.



**Figure 6: A role for E2F-1 in the control of proliferation, apoptosis and DNA repair:** (a) Under normal circumstances, E2F-1 regulates the expression of genes required for cell cycle progression (b) In response to DNA damage, E2F-1 is phosphorylated by ATM/ATR and Chk2 kinases, resulting in E2F-1 stabilisation. The high levels and/or modification of E2F-1 protein reached in response to DNA damage may allow the binding and activation of a different spectrum of genes involved in the induction of apoptosis (c) E2F-1 plays additional roles, perhaps independently of its transcription factor activity, in DNA damage repair and replication control (Stevens & La Thangue, 2004) .



**Figure 7: Domain organisation of E2F-1.** Serine residue 31 (S31) phosphorylated by ATM/ATR, and serine residue 364 (S364) phosphorylated by Chk2 kinase in response to DNA damage are indicated. E2F-1 has an N-terminal cyclinA/Cdk binding site, a nuclear localisation signal (NLS), and a binding site for the F-box protein Skp2, which is the cell cycle regulated component of the ubiquitin protein ligase SCF<sup>Skp2</sup>. The pocket-protein binding and *trans*-activation domain are located in the C-terminus. The position of the DNA binding, dimerization and marked box domains are also shown. The dimerization domain allows E2F-1 to interact with its heterodimeric partner, DP-1 (Stevens & La Thangue, 2004).

Nevertheless, the DNA damage responsive phosphorylation of E2F-1 has been clearly connected with increased E2F-1 protein stability and the induction of apoptosis, in a similar fashion to the control of p53 (Lin et al., 2001; Stevens et al., 2003). Given the established role of p53 in the DNA damage response and checkpoint control, it is highly likely that E2F-1 plays a similar role in checkpoint control.

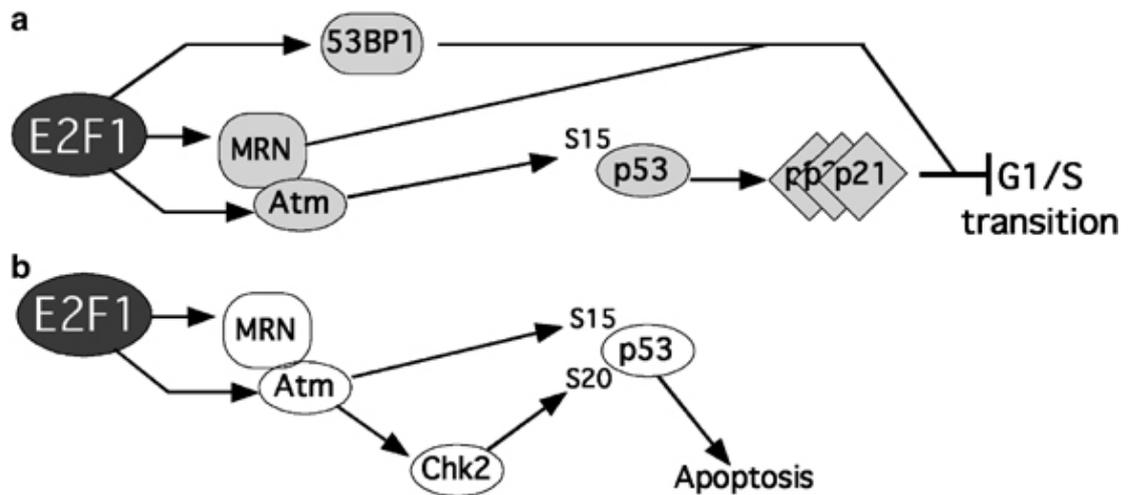
The DNA damage-induced phosphorylation of p53 has been shown to promote the subsequent acetylation of lysine residues within its carboxy-terminal region, which enhances p53 DNA-binding and transcriptional activity (Sakaguchi et al., 1998). The phosphorylation of E2F-1 by ATM/ATR and Chk2 kinases may promote a similar outcome, and indeed the acetylation of E2F-1 has been connected with phosphorylation by ATM/ATR kinases (Pediconi et al., 2003). Degradation via the proteasome requires that proteins are first targeted for destruction by the ubiquitination of lysine residues (Ciechanover et al., 2000). Thus, it is tempting to speculate that the increase in E2F-1 stability observed in response to DNA damage could be the result of a phosphorylation-acetylation cascade that prevents the normal turnover of E2F-1.

Several studies demonstrate that immediately following DNA damage induced by IR or genotoxic drugs, DNA damage proteins relocalize to sites of the double-stranded DNA breaks, for example, the MRN complex, 53BP1 (Nelms et al., 1998; Shiloh, 2003). A consequence of these events is the activation of a cell cycle check point pathway through p53/p21<sup>CIP</sup> that is dominant to E2F1-mediated apoptosis (Figure 8). To date, three pathways of E2F1 induced apoptosis has been documented, which are (1) a p53-dependent pathways through stabilization of p53 by E2F1; (2) p53-independent pathways through direct upregulation of apoptotic related genes

by E2F1; (3) blocking of antiapoptotic pathways through downregulation or inhibition of antiapoptotic molecules (Bell & Ryan, 2004).

Whilst DNA damage provides a signal for E2F-1 to induce apoptosis, the mechanism through which E2F-1 causes apoptosis rather than cell cycle progression remains unclear. It has been suggested that different target promoters may be occupied by distinct E2F subunits, for example as cells progress through the cycle, but to date there is little evidence to support any promoter-specificity of E2F complexes (Takahashi et al., 2000). However, a recent study investigated E2F-1 gene specificity during the DNA damage response, observing that apoptosis-inducing genes like p73 rather than cell cycle genes were specifically targeted by E2F-1 upon DNA damage (Pediconi et al., 2003). This gene specificity was connected with an increased level of E2F-1 acetylation, and required the integrity of the S31 ATM/ATR kinase site (Pediconi et al., 2003). It is possible therefore that the DNA damage regulation of E2F-1 alters target gene specificity in such a fashion that apoptosis-inducing genes are favoured, and that the acetylation of E2F-1 plays an intimate role in this process.

Against this background, it remains possible that the level of E2F-1 protein influences the effects in cells. For example, a threshold E2F-1 level, such as the high level reached in response to DNA damage, could determine a particular outcome, perhaps by allowing a different spectrum of genes to be activated. An equally valid model suggests that DNA damage and subsequent phosphorylation promotes the interaction of E2F-1 with accessory proteins, resulting in the activation of apoptotic target genes.



**Figure 8: Model of the relationship between E2F1-mediated check point and apoptosis pathway.** (a) Dominant pathway leading to a cell cycle check point. (b) Apoptosis signaling (Note that a subset of factors function in both pathways) (Frame et al., 2006).

When phosphorylated by Chk2, E2F-1 localises to distinct nuclear bodies with intense staining (Stevens et al., 2003). In this respect it is known that many proteins involved in the recognition and repair of DNA damage, including several activated by Chk2, localise to distinct nuclear foci most probably at sites of DNA damage (Rouse & Jackson, 2002). However, it is unclear if this location is connected with the apoptotic activity of E2F-1 or some other property of E2F-1 related to checkpoint control or DNA damage, although it is attractive to speculate that E2F-1 may have an additional role in the detection of DNA damage and, perhaps, its subsequent repair ( Figure **6c**). In support of this idea, the Mre11–Nbs1–Rad50 checkpoint protein complex involved in responses to DNA double stranded breaks (D'Amours & Jackson, 2002) associates with E2F-1 through the N-terminal region of Nbs1 (Maser et al., 2001). Furthermore, the Nbs1/E2F-1 interaction occurs close to these sites, suggesting that E2F-1 is required to target the Mre11 complex to origins of replication (Maser et al., 2001). Moreover, since *Drosophila* E2F and DP are present in origin recognition complexes (Bosco et al., 2001). It is possible that mammalian E2F-1 influences DNA replication upon DNA damage.

A recent study demonstrated that the DNA topoisomerase II $\beta$  binding protein 1 (TopBP1) relocalises with E2F-1 to nuclear foci along with BRCA1 in response to DNA damage (Liu et al., 2003). The recruitment of E2F-1 to foci along with the BRCA1-repair complex and TopBP1 further supports the idea that E2F-1 is involved in DNA damage repair and replication control. Combined with the earlier discussion, these results imply that E2F-1 is a multi-functional damage responsive protein that is involved in numerous aspects of the DNA damage response.

### **3. Thesis aims**

The objective of this dissertation was to non-invasively assess E2F-1 dependent transcriptional regulation *in vitro and in vivo*.

It is known that frequently deregulation of the E2F-1 transcription factor via alteration of the p16-cyclinD-Rb pathway is a key event in the malignant progression of human gliomas. Several recent reports have described that the E2F-1 transcription factor serves as a link between Rb/E2F proliferation pathway and the p53 signaling pathway. In response to DNA damage, E2F-1 protein level is increased and induces p53 signaling. This leads to either cell cycle arrest followed by DNA repair mechanisms or apoptosis. In this regard, the overall goal of investigation was:

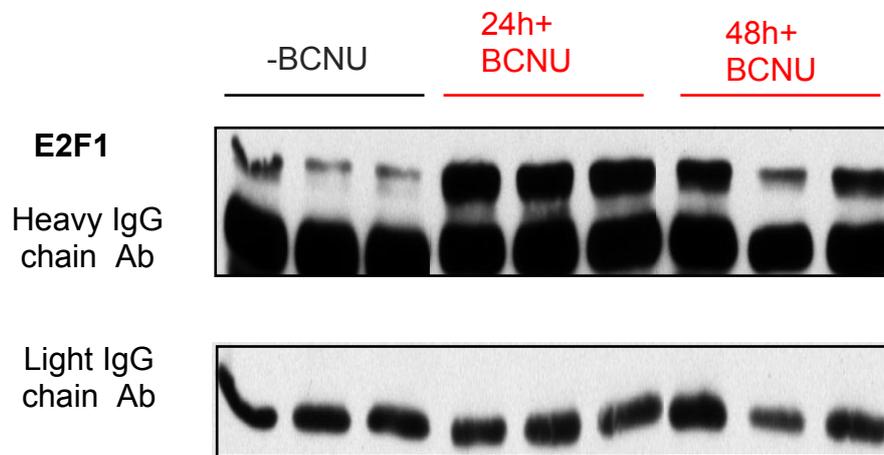
- Aim 1: To identify the basal level of E2F-1 expression following treatment with BCNU in human U87dEGFR glioma cell line.
- Aim 2: To generate a self-inactivating retroviral vector which carrying *Cis*-E2F-TA-LITG reporter system.
- Aim 3: To analyze the effect of BCNU in mediating E2F-1/ p53/ p21 signaling and blocking cell cycle progression.
- Aim 4: To study the efficacy of the *Cis*-E2F-TA-LITG reporter system by bioluminescent imaging *in vitro and in vivo*.

Due to the importance of E2F in the regulation of cell cycle progression and their deregulation in cancer, these analyses could provide us the feasibility of non-invasive monitoring of the kinetics of E2F-1-dependent reporter gene expression *in vitro and in vivo*.

## 4. Results

### 4.1 E2F-1 expression in U87dEGFR cells is altered in response to BCNU treatment

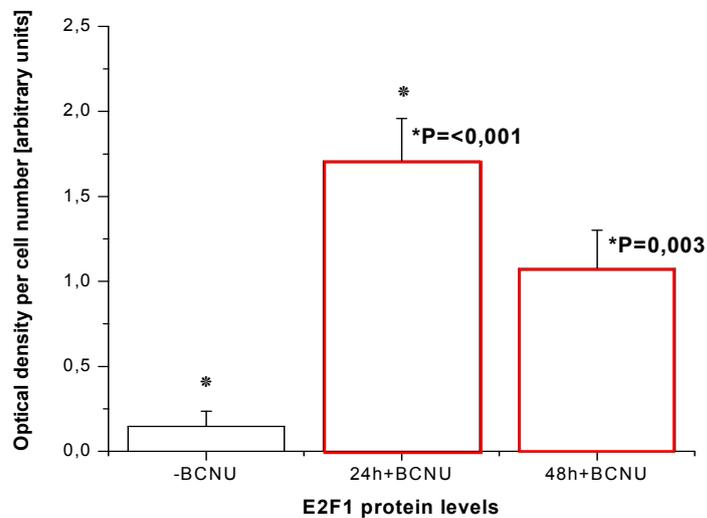
After DNA damage, one consequence of a deregulated pRb/E2F pathway is an increase in E2F-1 protein level. To verify this in the human U87dEGFR glioma cell line, we measured the E2F-1 protein levels in cell extracts prepared from U87dEGFR cells after BCNU treatment by immunoprecipitation and Western blot. Proliferating U87dEGFR cells were exposed to BCNU (25  $\mu$ M) and harvested 24h and 48h later. E2F-1 protein was immunoprecipitated by using monoclonal E2F-1 antibody and protein A/G-agarose. The E2F-1 protein level was checked by immunoblot analysis. As observed in **Figure 1A**, untreated cells show a basal level of E2F-1 expression which increased after exposure to BCNU with a maximum at 24 hours. The densitometric quantification of E2F-1 expression as shown in **Figure 1B** demonstrate the quantitative increase relative to the number of U87dEGFR cells for three independent experiments. These results indicate that BCNU treatment has a significant positive effect on the E2F-1 protein level in U87dEGFR cells ( $p < 0.001$ ; Student *t* test).



**Figure 1:**

**(A) A substantial induction of E2F-1 protein level in response to BCNU treatment**

The E2F-1 protein level was shown in non-treated and BCNU treated human U87dGEFR glioma cells at different incubation times. The primary anti-E2F-1 antibody recognizes E2F-1 protein at approx. 60 kDa (first band). The 55 kDa and 27 kDa heavy and light IgG chains, respectively, of the primary antibody were also detected.



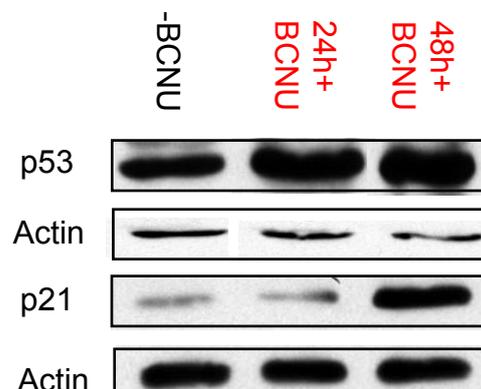
**Figure 1:**

**(B) A significant positive effect of BCNU on the E2F-1 protein level in U87dEGFR cells**

The E2F-1 protein level was normalized with respect to cell number. Significant differences between BCNU-treated and non-treated cells ( $P < 0.001$ ; Student  $t$  test) are indicated by \*. Error bars represent standard deviation. The findings depicted in A and B represents the results of three independent experiments.

#### 4.2 BCNU induces p53 and p21 expression in U87dEGFR cells

Given the fact that p53 protein plays an important role in protecting cells from the effects of DNA damage, we also evaluated p53 and p21 protein level in response to BCNU exposure by Western blot analysis. As shown in **Figure 2**, p53 and p21 levels increased in treated cells compare to non-treated cells with a maximum at 24h (p53) and 48h (p21 as downstream target for p53) following exposure to 25 $\mu$ M BCNU. Therefore we suspect that in response to treatment E2F-1 expression results in an increase in endogenous p53 protein levels. Activation of p53 may leads in activation of a p21-dependent cell cycle checkpoint.



**Figure 2:**

#### Induction of p53 and p21 protein levels in response to BCNU treatment.

Western blot analysis of p53 and p21 levels in non-treated and BCNU treated U87dEGFR cells. The level of the actin protein is shown as a loading control. Note that, as expected, p53, p21 and actin were recognized by antibodies at approximately 50, 20, and 40 kDa, respectively.

### 4.3 Characterization of E2F-1 regulated lucIREStkgfp expression in cell culture

To study drug dose- and time-dependent variations of luciferase gene expression in cell culture, U87dEGFR cell lines stably expressing *firefly luciferase (luc)* were engineered from different reporter vectors. The generation of reporter vectors is described in Materials and Methods.

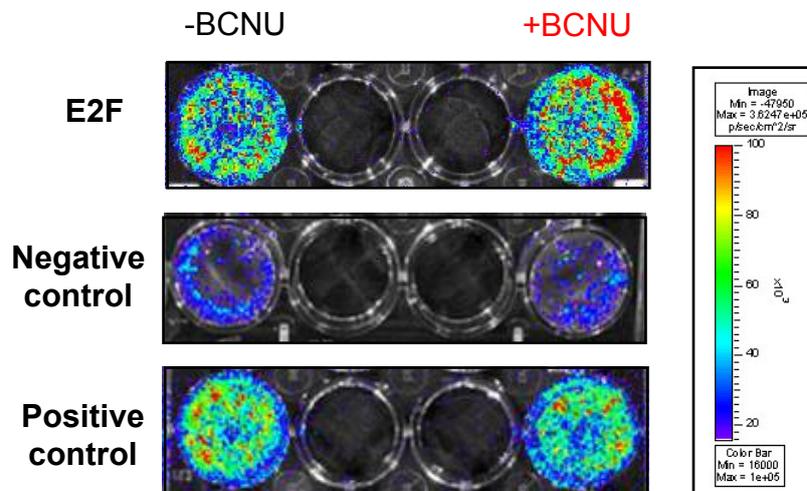
U87dEGFR cells expressing *lucIREStkgfp* (LITG) under transcriptional control of an E2F-1 responsive promoter (U87dEGFR-E2F-TALITG = test), minimal TA promoter (U87dEGFR-TA-LITG = non-regulated negative control), and LTR promoter (U87dEGFR-LITG = positive control), respectively, were seeded and treated with and without BCNU. Luciferin was added directly to the cell culture medium at different time points, and luciferase activity was monitored using the IVIS imaging system (**Figure 3A**). The light intensity was higher in U87dEGFR-E2F-TALITG cells as compared to non-regulated U87dEGFR-TA-LITG negative control cells. After BCNU treatment the bioluminescence signal intensity increased in U87dEGFR-E2F-TA-LITG cells whereas no obvious change was observed between non-treated and BCNU-treated negative and positive control cells (**Figure 3A**).

For quantitative analysis of different levels of LITG expression (luciferase component) in response to BCNU a microplate reader was used. A 3.6-fold difference was observed in U87dEGFR-E2F-TALITG cells as compared with non-regulated U87dEGFR-TA-LITG negative control cells with respect to the basal bioluminescent signal (**Figure 3B**). In U87dEGFR-E2F-TALITG cells the expression of LITG increased

4.0-fold in response to BCNU exposure whereas no significant increase was observed in negative (U87dEGFR-TA-LITG) and positive (U87dEGFR-LITG) control cells (**Figure 3B**). These findings were confirmed by quantification of the relative green fluorescence signal per single cell using the MPI-tool imaging software (MPI, Cologne, Germany)<sup>4</sup> showing a significant increase (1.9-fold) of green fluorescence in U87dEGFR-E2F-TA-LITG cells in response to BCNU treatment (**Figure 3C**).

The co-localization of IRES-mediated co-regulated LUC and TKGFP protein co-expression in cells carrying the E2F regulated LITG construct is depicted in **Figure 3D**.

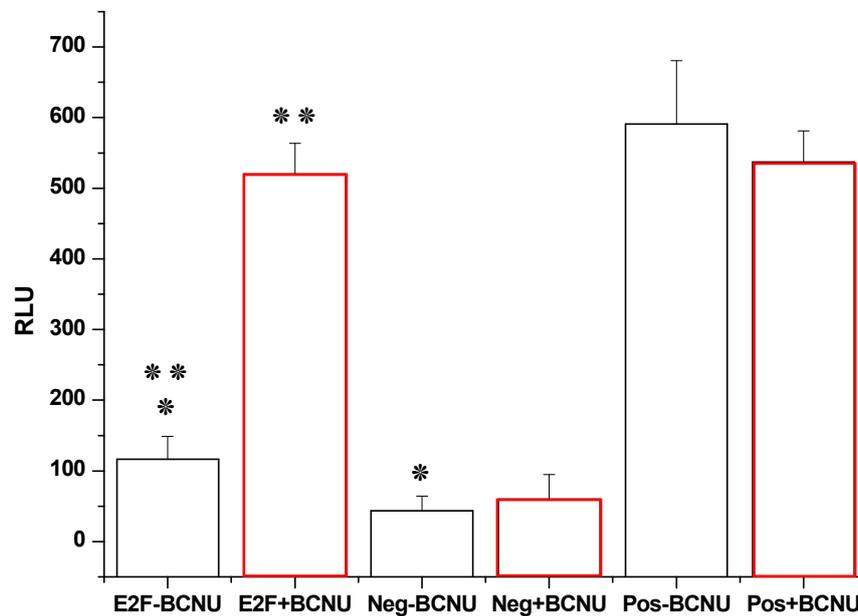
Taken together, these data indicate that the light production from E2F stably expressing cells correlate with other indicators of cell cycle progression and DNA damage signals.



**Figure 3:**

**(A) Bioluminescence imaging of E2F transcriptional activity induced in cell culture**

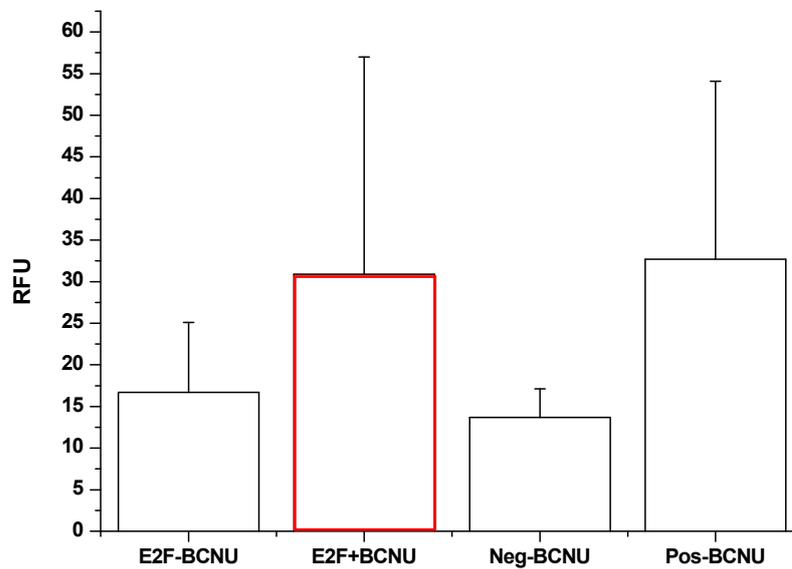
E2F-responsive transcriptional activity was imaged by the IVIS imaging system. Measurements were made in U87dEGFR-E2F-TA-LITG glioma cells (first row) in comparison to U87dEGFR-TA-LITG (middle row) and U87dEGFR-LITG (third row) glioma cells.



**Figure 3:**

**(B) Positive effect of BCNU on the Bioluminescence output related to E2F transcriptional activity**

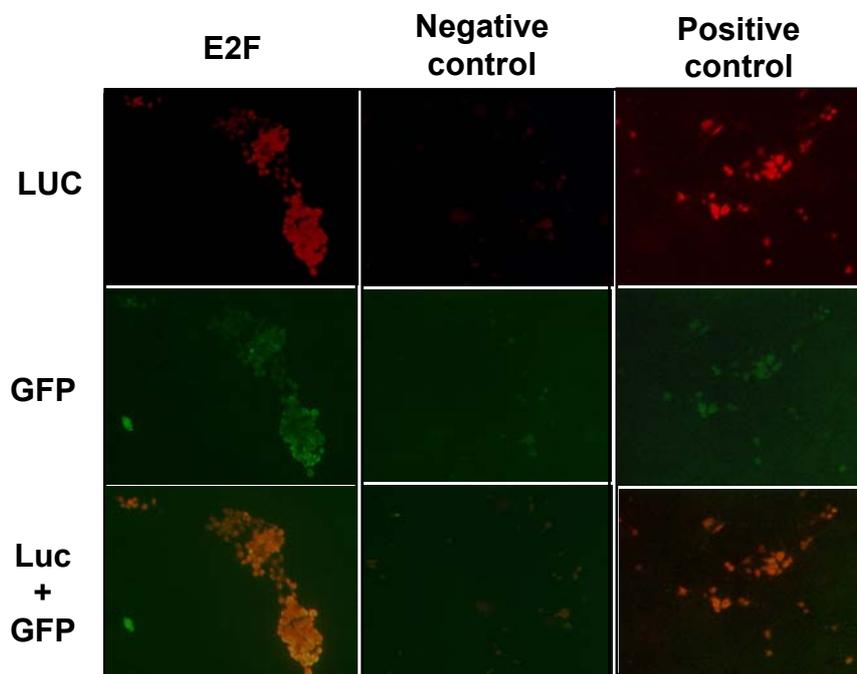
Relative E2F transcriptional activity is quantitated by microplate reader. U87dEGFR-E2F-TA-LITG cells have a significantly higher signal in comparison to negative control U87dEGFR-TA-LITG cells (t-test;  $P=0.029$ ). After BCNU treatment, luciferase signal increases significantly in E2F-regulated U87dEGFR-E2F-TA-LITG cells (t-test;  $P<0.001$ ), but not in negative and positive control cells.



**Figure 3:**

**(C) Intensity of green fluorescence in response to BCNU correlates with E2F activity in response to BCNU treatment.**

Relative intensity of green fluorescence as recorded in single cells by mean of a ROI analysis using MPI-tool imaging software<sup>4</sup> indicating again a significant induction of luciferase signal after BCNU treatment in E2F-1-regulated U87dEGFR-E2F-TA-LITG cells.



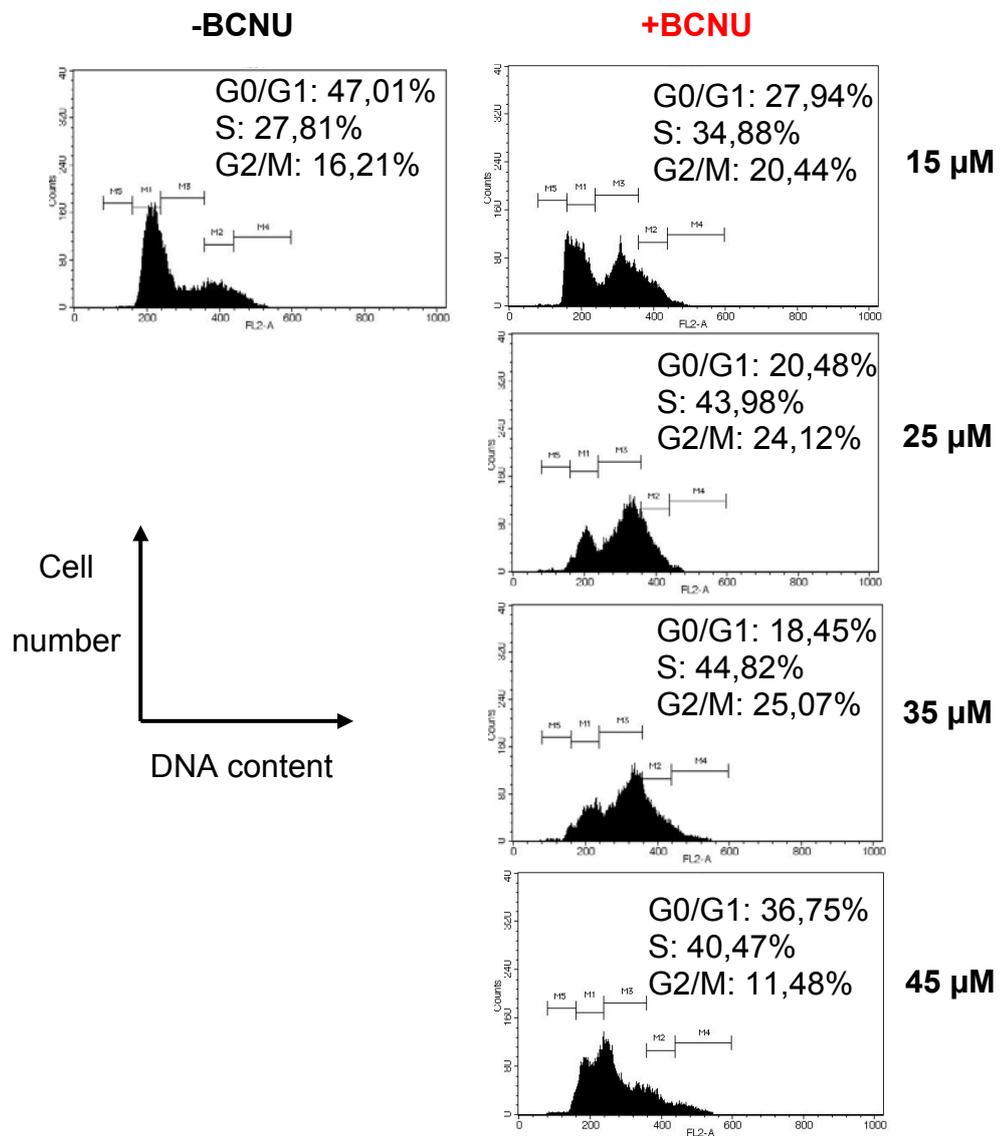
**Figure 3:**

**(D) Assessment of the *Cis*-E2F-TA-LITG reporter system by immunofluorescence microscopy**

Co-localization of LUC and GFP in U87dEGFR-E2F-TA-LITG cells as compared with non-regulated negative (U87dEGFR-TA-LITG) and positive (U87dEGFR-LITG) control cells, U87dEGFR cells stably expressing luciferase were subjected with anti-LUC and fluorescence microscopy.

#### **4.4 BCNU treatment leads an arrest in dose dependency manner**

Cell cycle arrest is a common response to DNA damaging agents. Given the up-regulation of E2F-1 in response to BCNU treatment and the role of E2F-1 in cell cycle regulation, we examined the possible contribution of alkylation-induced arrest on cell cycle progression using flow cytometry. We evaluated the cell cycle distributions of U87dEGFR-E2F-TA-LITG cells following exposure to different doses of BCNU. As shown in **Figure 4**, 24h after exposure there was a dramatic increase in S-phase populations in treated cells compared with non-treated cells. In addition, a substantially increase in G1-phase was only observed at the highest does of BCNU. Accumulation in S/G2 phase following BCNU exposure suggests that significant proportion of cells were unable to complete DNA synthesis and continue with cell division. Likewise, Cells gave stronger response at the highest does of BCNU.

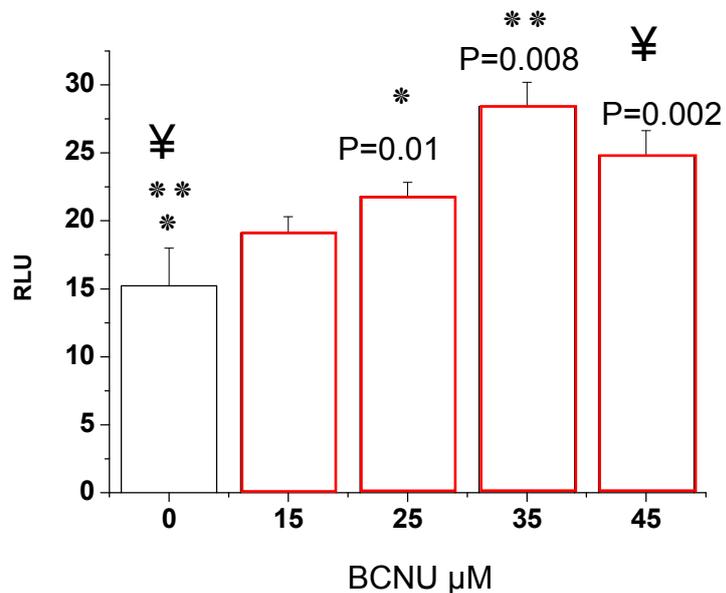


**Figure 4:**  
Graphs depict cell cycle distributions of U87dEGFR-E2F-TA-LITG cells.

Cell cycle distributions were underwent sustained S/G2-phase arrest following treatment with different concentrations of BCNU. Of note, a greater proportion of cells accumulated in G1-phase at the highest BCNU concentration.

#### 4.5 BCNU treatment induces apoptosis

To further investigation, the effect of BCNU in induction of caspase-mediated apoptosis was evaluated by measuring the activation of caspases- 3/7 in cells treated with BCNU. The semiconfluent monolayer of cells was treated with BCNU for 24h to measure the induction of caspase-3/7. Although BCNU increased the activities of caspase-3/7 in cells with different concentrations of BCNU, the activation of caspase-3/7 was significantly more prominent from 25  $\mu$ M BCNU concentrations. The caspase-3/7 activity in cells treated with the 45  $\mu$ M concentration was lower, probably due to the cytotoxic effect of BCNU at this concentration (**Figure 5**).

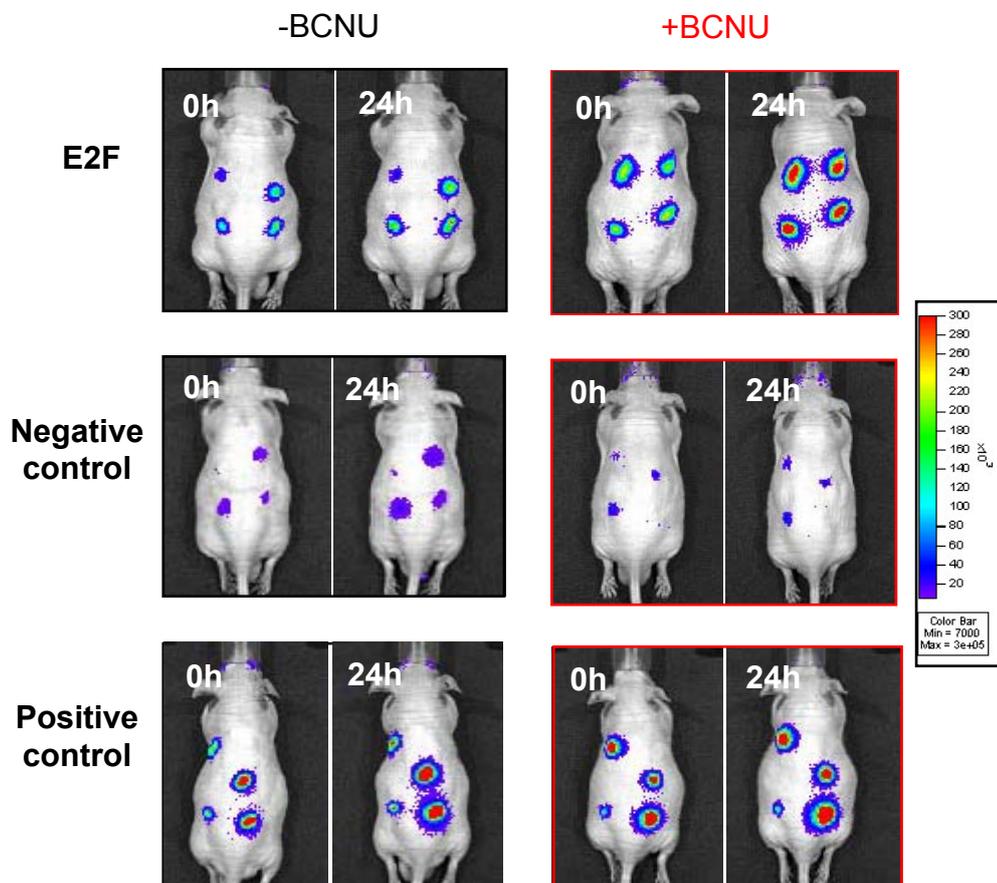


**Figure 5:**  
**The effect of BCNU (15-45 μM) on caspase-3 activity in U87dEGFR-E2F-TA-LITG cells**

The activities of caspase-3/7 were measured using the Caspase-Glo 3/7 kit (Promega Corp., Madison, WI) in U87dEGFR-E2F-TA-LITG cells. Cells were cultured in 96-well plates until they achieved semiconfluency and then treated with the indicated concentrations of BCNU for 24h. The Caspase-Glo 3/7 reagent (100 μl) was added directly to the wells and plates were incubated for 1h before recording the luminescence reading. Each value represents the average of at least three different values.

#### 4.6 Visualization of E2F-1 transcriptional activity *in vivo*

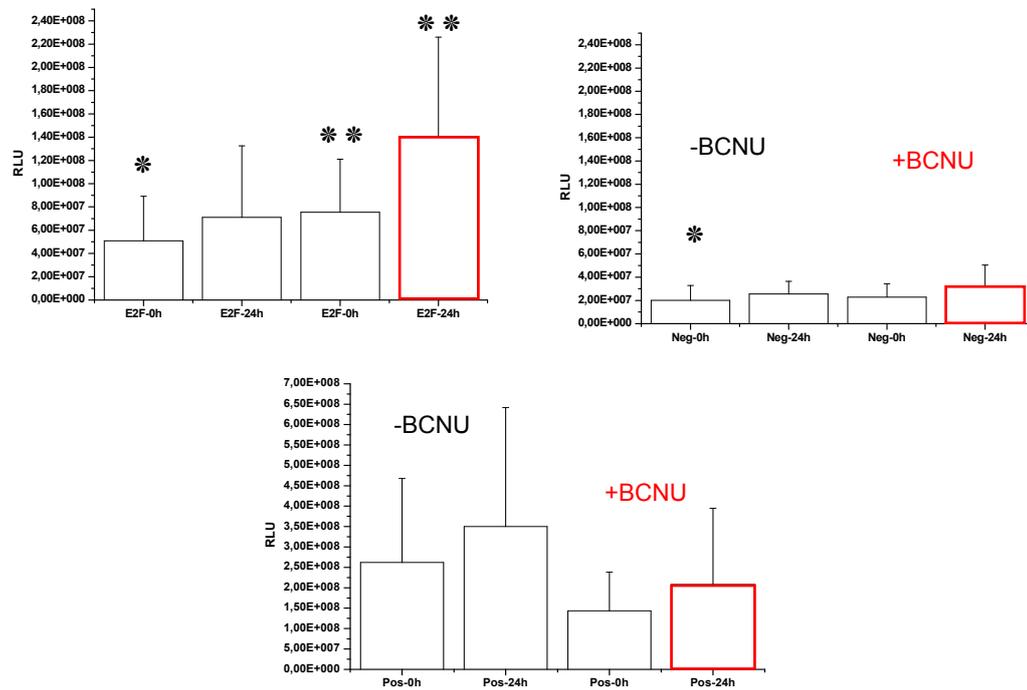
In order to assess the efficacy of the *Cis*-E2F-TA-LITG reporter system *in vivo*, each cell line was implanted subcutaneously into four sites of nude mice: U87dEGFR-E2F-TALITG (test; n=2), U87dEGFR-TA-LITG (non-regulated negative control; n=2), U87dEGFR-LITG (non-regulated positive control; n=2). Experiments were repeated twice resulting in n=6 animals per cell line. Seven days later, half of the animals were injected with BCNU (25 mg/kg, i.p.) to induce E2F activity in response to DNA damage. After intraperitoneal administration of luciferin, mice were imaged daily with the Xenogen IVIS system (**Figure 6A**). As expected, the non-treated test mice bearing U87dEGFRE2F-TA-LITG gliomas showed a 2.5-fold higher basal intensity of bioluminescence signal as compared to mice bearing non-regulated U87dEGFR-TA-LITG negative control gliomas. In BCNU-treated test mice, a 1.9-fold induction of bioluminescence intensity was observed 24h after treatment with BCNU. No significant changes were found either in non-treated test mice or in negative and positive controls mice (**Figure 6B**). These results indicate that the activity of endogenous E2F-1 protein and its regulation by an exogenous stimulus (BCNU) in glioma cells can be detected and quantified noninvasively *in vivo*.



**Figure 6:**

**(A) Bioluminescence imaging of endogenous E2F-1 *in vivo***

The colour scale represents luminescent signal intensity, with blue indicating the least intense and red the most intense light. BLI signal increases after addition of BCNU in mice bearing U87dEGFR-E2F-TA-LITG gliomas (first row). Negative control mice bearing U87dEGFR-TA-LITG gliomas exhibit background signal only (second row). Positive control mice bearing U87dEGFR-LITG glioma exhibit high BLI signal independent on BCNU treatment.



**Figure 6:**

**(B) Mean of the total bioluminescent signals emitted from s.c. tumors in response to BCNU administration.**

Data are representative of 3 independent experiments with  $n=6$  per group. Upper- left graph represents data from mice bearing E2F-1 responsive U87dEGFR-E2F-TA-LITG gliomas. Upper- right graph represents data from mice bearing U87dEGFR-TA-LITG negative control gliomas. Lower graph represents data from mice bearing U87dEGFR-LITG positive control gliomas. Significant differences (Student  $t$  test;  $P=0.01$ ) are depicted by \*.

## 5. Discussion

The data presented in this thesis demonstrate the feasibility to non-invasively assess E2F-1-dependent transcriptional regulation *in vitro* and *in vivo*. Indeed, we were able to study various levels of E2F-1 expression at base line and after exposure to the DNA damaging agent BCNU with its known effects on various cell cycle regulating proteins, such as E2F-1, p53 and p21. We demonstrate that different levels of endogenous E2F-1 activity can be quantified and distinguished non-invasively *in vivo* by molecular imaging technology. These findings have important implications for studies aiming towards the non-invasive assessment of altered expression of key cell cycle regulators in response to molecular targeted therapies.

Our data support the several recent reports describing the induction of E2F-1 in various cancer cell lines in response to various forms of DNA damage such as  $\gamma$ -irradiation, UV-irradiation, and other chemotherapeutic drugs (Blattner et al., 1999; Hofferer et al., 1999; Huang et al., 1997; Lin et al., 2001; Meng et al., 1999). For example DNA damage caused by BCNU, an agent which intercalates into DNA, resulting in DNA strand breaks. We also found that the E2F-1 protein level significantly increased 24 hours after treatment with BCNU (**Figure 1 A, B**). Our data presented in **Figure 2** show that p53 and p21 expression were increased following BCNU treatment. These observations that BCNU treatment is leading to an increase expression of E2F-1, p53 and p21 suggest the contribution of E2F-1 in changing p53 protein level in response to BCNU. Recent studies also demonstrate that E2F-1 and p53 are cooperated in response to DNA damage by different mechanisms {Powers, 2004 #27; Hershko, 2005 #29}. This may involve a number parallel and perhaps synergistic mechanism. For example, a pathway,

links integration of E2F1 signaling and activation of the Atm/Chk2/p53 pathway, offers a mechanism for the proposed involvement of E2F-1 and p53 resulting from DNA damage. The authors (Blattner et al., 1999; Hofferer et al., 1999; Huang et al., 1997; Lin et al., 2001; O'Connor & Lu, 2000) argue that following treatment of cells with DNA damaging agents, E2F-1 protein accumulates and is phosphorylated at an N-terminal Atm recognition sequence that is unique to E2F-1 among the E2F family members. This phosphorylation of E2F1 is largely dependent on Atm and is required for efficient E2F1 stabilization following DNA damage. Chk2 has also been shown to phosphorylate and stabilize E2F1 following DNA damage. Accordingly, additional studies are required to fully elucidate the combinational effects of different mechanisms and the intricate network by which E2F-1 affects the level and the activity of p53 in response to BCNU treatment in U87dEGFR-E2F-TA-LITG cells.

Since an increasing number of reports indicate a so called “yin and yang” activity of E2F1 function, it is now obvious that E2F-1 plays a critical role in cell cycle arrest and apoptosis (DeGregori & Johnson, 2006; Johnson & Degregori, 2006; Stevens & La Thangue, 2004). Therefore, the aim of our work was to establish a model system to assess various levels of E2F-1 activity in the living model *in vivo* to serve an improved understanding of the *in vivo* kinetics of the cellular response to growth-altering or deathinducing agents. To do so, we created an E2F-1 regulated expression system to allow the non-invasive monitoring of E2F-1 regulated reporter gene (LITG) expression. While a recent paper has shown the possibility to image p53 transcriptional activity in tumor xenografts by using the *Cis*-p53TKGFP reporter system (Dobrovic et al., 2001), we generated a lucIREStkgfp (LITG) reporter system, where expression of LITG is controlled by an E2F-1

responsive element. We constructed and tested two self-inactivating retroviral vectors bearing the E2F-TA- (test) and a TA- (non-regulated negative control) promoter, respectively, controlling the expression of the LITG reporter construct. A third retroviral vector bearing LITG under control of the 3'LTR promoter served as positive control (**material and methods**). These vectors were used to establish human U87dEGFR glioma cells stably expressing these expression systems. To evaluate the E2F-1-sensitive reporter system, we analysed nontreated and BCNU-treated U87dEGFR cells expressing the different constructs at different time points using various independent assays: fluorescence microscopy, immuno-cytochemistry, and quantification of luciferase activity and TKGFP expression (**Figure 3A-D**) flow cytometry and caspase-3 activity (**Figure 4, 5**). We demonstrate that the *Cis*-E2F-TA-LITG construct is sufficiently sensitive to monitor upregulation of E2F-1 expression following treatment with BCNU in U87dEGFR-E2F-TA-LITG cells *in culture* and with these results being in agreement with western blot analysis where E2F-1 protein levels were significantly increased 24 hours after treatment with BCNU.

A block in cell cycle progression is commonly observed following DNA damage when a cell cycle checkpoint is activated to prevent replication of damaged DNA (Caspari & Carr, 2002). G2 arrest has been noted previously in various cell types in response to the nitrosourea (Tobey, 1975; Tobey & Crissman, 1975), including BCNU (Hoshino et al., 1981) and in response to some other alkylating agents (Tobey, 1975). Our data implicates that BCNU caused two different types of growth inhibition in U87dEGFR-E2F-TA-LITG cells (**Figure 4**). At the low concentrations, there was an accumulation of cells in S/G2 phase and a decrease in the G1 population, suggesting mitotic arrest at these lower doses. At a high concentration, rapid accumulation of cells in

G1 phase occurred within 24 hours. The difference in the effect of BCNU on cells at the different concentrations suggests that there may be two distinct cytotoxic mechanisms for BCNU at low and at high dosages. Together these observations raise the possibility that E2F-1 activation by DNA damage leads to block cell cycle progression in part through its ability of activate p53 resulting in accumulation of p21 protein. We hypothesized that E2F-1 is activating a cell cycle checkpoint prior to the induction of apoptosis. It remains to be determined if there are differences in p53 activation by E2F-1 that mediate either a growth arrest or apoptosis.

Due to the fact that treatment with low concentrations of BCNU eventually caused growth inhibition and probably also cell death in treated cells, a significant caspase-3 activity was not detected with low concentrations of BCNU. It appears that treatment with low concentrations of BCNU initiated a process that gradually but irreversibly brings the cells to death, probably through inhibiting cell proliferation. In contrast, apoptosis occurred rapidly in cells treated with high concentrations of BCNU (**Figure 5**).

We speculate that E2F1 may initiate a signalling cascade to activate a cell cycle checkpoint and induce a block in cell cycle progression associated with redistribution of DNA damage repair molecules. If proper cell cycle regulation is not returned, sustained E2F1 activity would then promote apoptosis.

Clinical dosages of BCNU are often limited by toxicity to other organ systems, and the actual drug concentrations delivered to tumor sites *in vivo* may be considerably lower than the levels that induce apoptotic pathway. However, there is a growing body of evidence showing that deregulation of E2F transcription factors is also causatively involved in carcinogenesis, and several members of the E2F family are associated with clinical outcome in

various cancer entities. Moreover, our observations clearly argue that, under certain circumstances, E2F-1 can also be involved in apoptosis by targeting key genes in the p53 pathway. We therefore investigated to assess quantitatively treatment efficacy and the expression of E2F-1 that is cis-linked to the reporter genes *in vivo*. We assumed that this may offer a better understanding of the expression dependent on cell proliferation and apoptosis especially as related to E2F-1 activity.

Most importantly, we have demonstrated that our reporter system is sufficiently sensitive for visualization of E2F-1 activity using bioluminescence imaging *in vivo* (**Figure 6A-B**). Indeed, the up-regulation of E2F signaling pathway and luciferase gene expression can be quantified. These observations can be supported by direct analysis of tissue samples. To our knowledge, this is the first study of cis- acting enhancer reporter system that is distinguishing the activity of enhancer element with the basal viral promoter activity.

To conclude, these combined *in culture* and *in vivo* results demonstrate that the images of the *Cis*-E2F-TA-LITG reporter system obtained by BLI reflect the kinetic changes of the E2F-1 signalling pathway.

It should be pointed out that recent studies have used E2F-1 apoptotic target genes as new therapeutic drug targets thereby providing insight into the basic mechanisms of E2F-1-induced apoptosis and its possible clinical implications. For example, most tumors lack functional p53 and, therefore, are resistant to conventional therapeutic treatment (Hanahan & Weinberg, 2000). The E2F-1 target genes, especially those that induce apoptosis independently of p53 (i.e. p73) (Pediconi et al., 2003), may give rise to the development of new forms of molecular therapeutic intervention (Braithwaite et al., 2006; Jamshidi-Parsian et al., 2005; Stanelle & Putzer, 2006). In the

past few years, preclinical experiments using mainly E2F-1 as an anti-cancer therapy have been initiated. The effect of E2F-1 overexpression on tumor growth has been evaluated in several types of human cancer. These studies have clearly indicated that induction of apoptosis by overexpression of E2F-1 via adenoviral-mediated gene transfer results in tumor growth suppression. Moreover, E2F-1 was shown to sensitize tumor cells to chemotherapy (Dong et al., 1999; Fueyo et al., 1998; Kaelin, 2003; Liu et al., 1999). A study by Fine et al. described an E2F-1-responsive adenoviral vector for gliomas that promises to target cancer cells more specifically than the standard approach, thereby reducing the therapy's toxicity to normal tissue (Parr et al., 1997). Since the brain is the central organ to control body function and mind, developing improved methods to selectively kill malignant cells while preserving the surrounding neuronal tissue would be of considerable value.

In summary, the *Cis*-E2F-TA-LITG reporter system allows the non-invasive monitoring of the kinetics of E2F-1-dependent reporter gene expression *in vivo*. The E2F-1 pathway is complex and impacts cellular growth, apoptosis, and response to various stimuli, such as DNA damage. Little is known about the E2F-1-dependent regulation *in vivo*, the most of our knowledge coming from studies of cultured cell lines. We propose that these types of reporter systems, such as *Cis*-E2F-TA-LITG, will allow a detailed insight into the kinetics of cell cycle regulating protein expression *in vivo* as the basis for the further understanding of cell cycle control and for the development of new cell cycle targeted molecular therapies.

While we have made significant advances toward our understanding of E2F1 signaling, a number of questions require further exploration. How does

expression of E2F1 result in activation of DNA damage response factors? The finding that E2F1 may activates both a cell cycle checkpoint and an apoptotic response suggests that there may be a molecular switch between the divergent outcomes. How the cell decides which pathway to favor remains unclear. In addition, E2F-1 may function in multiple pathways in the cellular response to DNA damage. Lastly, the exact mechanisms that control E2F specificity remain to be determined. It is conceivable that deregulation of both proliferation –promoting and proliferation-inhibiting E2F transcription factors and their cross-talk is crucially involved in the tumor biology of brain cancer and influences clinical outcomes.

Further studies are warranted to determine the potential role of E2F family members in the postulated clinic effect especially as a putative predictor in this tumor entity.

## 6. Abstracts

### 6.1 Zusammenfassung

Die Deregulierung des E2F-1 Transkriptionsfaktors durch Umbildung des p16-cyclinD-Rb Signalweges gilt als wichtiges Ereignis beim malignen Fortschreiten der meisten humanen Gliomas. Paradoxerweise erfüllt E2F-1 die Funktion eines Tumor Supressors und eines Oncogens

Ziel dieser Arbeit war es, nicht-invasiv E2F-1 abhängige transkriptionale Regulation in vitro und in vivo nachweisen zu können. Zunächst wurde die E2F-1 Expression in U87 dEGFR Zellen nach Induzierung von DNA-Schäden mittels BCNU untersucht, die als Antwort auf DNA-Schädigungen ansteigt. Es konnte in unserem Model gezeigt werden, das der E2F-1 Level in diesen Zellen 24 Stunden nach Behandlung mit BCNU signifikant erhöht war. Außerdem kann E2F-1 nach DNA-Schädigungen p53 induzieren, was entweder eine Unterbrechung des Zellzyklus mit anschließender Aktivierung der DNA-Reparaturmechanismen, oder Apoptose zur Folge hat. Daher wurde der Effekt von BCNU auf den p53 Signalweg und die Unterbrechung des Zellzyklus untersucht. Die Analyse zeigte eine Rolle für p53 und E2F-1 in der Aktivierung eines p21 abhängigen Zellzyklus Kontrollpunktes im Vorfeld der Aktivierung von Apoptose. Um nachzuweisen, ob ektopische E2F-1 Expression nicht-invasiv mittels molekularer Bildgebung quantifiziert werden kann, wurde nun ein *Cis*-E2F-TA-LITG Reporter System generiert. Die in den folgenden Untersuchungen gewonnen Resultate zeigten die Anwendbarkeit und den Nutzen der nicht-invasiven Untersuchung der Kinetik der E2F-1 abhängigen Reporter Gen Expression in vitro und in vivo.

Die in dieser Arbeit gemachten Untersuchungen zeigen die Möglichkeit, mit Reportersystemen wie dem hier genutzten detaillierte Einsicht in die

Kinetik der Zellzykluskontrolle zu erhalten und neue, auf den Zellzyklus ausgerichtete molekulare Therapien zu entwickeln.

## 6.2 Abstract

There is strong evidence supporting the theory that deregulation of the E2F-1 transcription factor via alteration of the p16-cyclinD-Rb pathway is a key event in the malignant progression of most human gliomas. Paradoxically, E2F-1 behaves as both an oncogene and a tumor suppressor gene.

Our aim was to non-invasively assess E2F-1 dependent transcriptional regulation *in vitro and in vivo*. Due to the fact that E2F-1 protein level is increased in response to DNA damage, we tested the expression of E2F-1 in human U87dEGFR glioma cells after induced DNA damage by BCNU. We found that the amount of E2F-1 protein significantly increased 24 hours after treatment with BCNU. Moreover, E2F-1 can induce p53 following DNA damage leading to either cell cycle arrest followed by DNA repair mechanisms or apoptosis. Therefore, we analyzed the effect of BCNU in mediating p53 signaling and blocking cell cycle progression. The analysis revealed a role of E2F1 and p53 in activation of a p21-dependent cell cycle check point prior to the induction of apoptosis. To determine if ectopic E2F expression non-invasively can be quantified and distinguished by molecular imaging technology, we generated the *Cis*-E2F-TA-LITG reporter system. Our observations address the feasibility of non-invasive monitoring of the kinetics of E2F-1-dependent reporter gene expression *in vitro and in vivo*.

We propose that these types of reporter systems, will allow a detailed insight into the kinetics of cell cycle control and for the development of new cell cycle targeted molecular therapies.

## **7. Material and Methods**

### **7.1 Glioblastoma cells and culture conditions**

Human U87dEGFR glioma cells with functional wild-type p53 were obtained from Dr. W. Cavenee, Ludwig Ins. San Diego (Van Meir et al., 1994). The amphotrophic retroviral packaging cell line PHOENIX (kind gift of Dr. Nolan, Stanford University) was used for packaging of the retroviral plasmids into retrovirus vector particles. The cell lines were grown as monolayer in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; Roche Diagnostics, Mannheim, Germany) and 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere. Retrovirally transduced human U87dEGFR glioma cells were selected with 500 µg/ml G418 (PAA Laboratories GmbH, Cölbe, Germany). U87dEGFR-E2F-TA-LITG, U87dEGFR-TA-LITG, and U87dEGFR-LITG were obtained by selection in culture media supplemented with 2 µg/ml puromycin (Sigma, Taufkirchen, Germany).

### **7.2 Chemotherapeutic agent**

BCNU (*Mr* 214.06) was purchased from Bristol-Myers Pharmaceuticals (München, Germany). A stock solution was prepared by dissolving 100 mg of BCNU in 30 ml of 10% ethanol, and was stored at -70°C to ensure drug stability (Tepe et al., 1991). Subsequently, BCNU stock solution was added to culture medium or was injected intraperitoneally into experimental animals to achieve the desired concentrations for experimental use.

### **7.3 Immunoprecipitation**

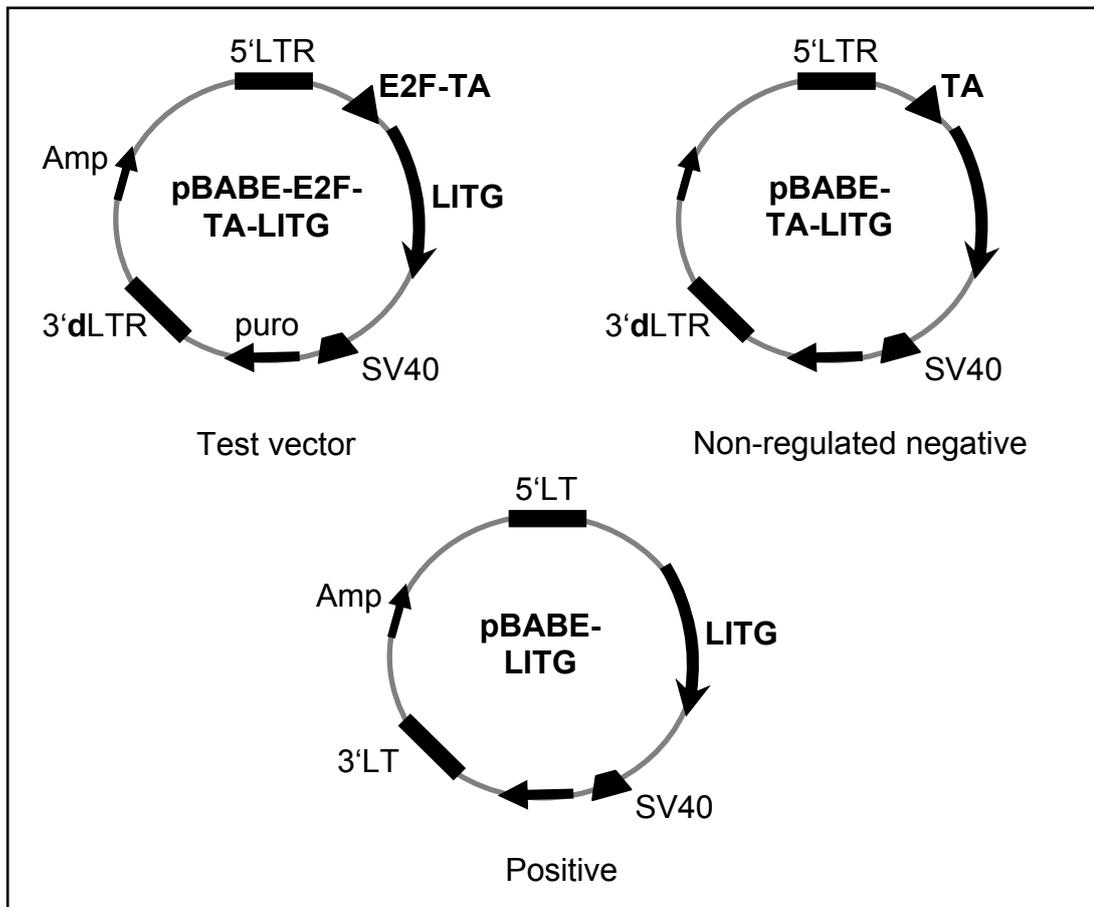
To estimate the amount of endogenous E2F-1 protein following treatment with BCNU, immunoprecipitation was performed. Total cell extracts were prepared 0, 24, and 48 hours after BCNU administration. After washing (ice cold PBS), cells were resuspended in lysis buffer (10mM Tris pH 8, 1 mM EDTA, 1mM DTT, 0.5% Triton) including s instructions. 48 hours'by the calcium phosphate method according to manufacturer proteinase inhibitor mix (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 30 min on ice. Samples were cleared by centrifugation, and supernatants were collected. Equal protein amounts were precleared with protein A/G-agarose (Santa cruz) for 1 h at 4°C under gentle rotation. Thereafter, immunoprecipitation was performed by adding monoclonal anti-E2F1 antibody KH95 (Santa Cruz; Heidelberg, Germany) and protein A/G-agarose at 4°C. Beads were washed three times (washing buffer) and bound proteins were subjected to Western blot analysis. In parallel, non-treated and BCNU-treated cells were harvested for determination of cell number as measured by a cell counter (Z1 particle counter Beckman, Coulter Krefeld, Germany).

#### **7.4 Western blot analyses**

The expression of E2F-1 in response to BCNU treatment was evaluated by Western blotting. Briefly, monolayers were harvested 24h and 48h after BCNU treatment and resuspended in phosphate buffered saline (PBS). Cells were lysed and the amount of protein present in each sample was quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of denatured (95°C, 5 min) protein were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. After separation (1 hr, 100 V) proteins were blotted to a Hybond-ECL membrane (Amersham Pharmacia Biotech, Freiburg, Germany) in standard Tris-glycin transfer buffer (100 V, 240 mA, 1.25 h). After blocking nonspecific binding (5% nonfat dry milk in 0.1% PBS-Tween20; 1 hr) the membrane was washed (0.1% PBS-Tween20) and then probed with primary antibodies: anti-E2F KH95 at 1:500 dilution, and anti-actin C-2 at 1:200 dilution (Santa Cruz, Heidelberg, Germany), respectively, in 5% dry milk and 0.1% PBS-Tween20 (1 hr). After washing, secondary antibodies were applied (Goat Anti-Mouse IgG [Southern Biotechnology Associates, Inc., Amersham Munich, Germany] with horseradish peroxidase conjugated 1:10.000 in 5% dry milk in 0.1% PBS Tween20; 1 hr). For final protein detection chemiluminescent reaction (Pierce Biotechnology, Inc., Schwerte, Germany) was used. PCBAS software was used to quantify the intensity of protein expression as assessed by Western blot.

### **7.5 Generation of reporter vectors**

The schematic structures of the retroviral vectors (pBABEpuro Moloney murine leukaemia virus-based vector backbone; kind gift of Dr. Miguel Sena-Esteves, Harvard Medical School, Boston, USA) are depicted in **Figure 1**. E2F1-TA firefly luciferase cDNA was amplified from pE2F-TA-Luc (test) and TA-Luc (negative control) from pTA-Luc (Clontech, Palo Alto, CA) using the forward primers 5'- TCT TAC TCG AGC TAG CCT TGG CG- 3', 5'-CGG GCT CGA GAT CTA GAC TCT AGA GGG-3' respectively and reverse primer 5'- CCG CGG ATC CTC TAG AAT TAC ACG GCG A-3'. XhoI (forward primer), and BamHI (reverse primer) sites were incorporated into the primers to facilitate cloning of fragments. The PCR products (E2F1-TA-luc and TA-luc) were digested with XhoI and BamHI and replaced upstream of the TKGFP gene into similarly cut pBABE-Neo-IRESTKGFP to generate pBABEneo-E2F-TA-LITG and pBABEneo-TA-LITG, respectively. A positive clone was confirmed by restriction enzyme digestion and DNA nucleotide sequencing. Thereafter, the neomycin resistance gene in these vectors was exchanged to the puromycin resistance gene using SfiI/NheI restriction sites to generate pBABEpuro-E2F-TA-LITG, and pBABEpuro-TA-LITG retroviral vector plasmids. To assess the transcriptional activity of the human E2F promoter and TA promoter, the self-inactivating feature of the vector was provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR enhancer sequences.



**Structure of retroviral vectors bearing *Cis*-E2F-TA-LITG, TA-LITG, and LITG.** In **pBABE-E2F-TA-LITG**. The expression of the luc<sub>1</sub>REStkgfp gene is regulated by an artificial promoter containing four copies of the E2F enhancer element, located upstream of the minimal TA promoter, the TATA box from the herpes simplex virus type 1 thymidine kinase promoter. Constitutive expression of the puromycin-resistance gene (*puro*) is driven by the simian virus 40 immediate early promoter, allowing for the selection of stably transduced cells. Moreover, this vector has a mutation in the 3'LTR (3'dLTR) that renders the silencing of its promoter activity after duplication as 5'LTR during integration. pBABE-TA-LITG lacks the E2F responsive element and serves as non-regulated negative control. pBABE-LITG bears an intact 3'LTR and serves as positive control.

## 7.6 Packaging and transduction of tumor cell line

The packaging of pBABEpuro derived plasmids bearing *Cis*-E2F-TA-LITG, TA-LITG and LITG into retrovirus particles was performed as described previously<sup>25,26</sup>. In brief, PHOENIX cells were grown as monolayers in 60 mm dishes and transfected with vector after transfection, the retrovirus containing medium was harvested, filtered (0.45µm), stored in aliquots at -80° for later use or supplemented with polybrene (8µg/ml) (Sigma, Taufkirchen, Germany) and used to infect proliferating human U87dEGFR glioma cells.

## 7.7 Selection of transduced tumor cell clones

To generate stable cell lines, 48 hours after infection U87dEGFR-E2F-TA-LITG, U87dEGFR-TA-LITG, and U87dEGFR-LITG glioma cells were washed, trypsinized, replated (1:10) and propagated under selection with puromycin (2 µg/ml) for 2 weeks.

## 7.8 Quantification of luciferase gene expression in cell culture

Cells were seeded at a concentration of  $5 \times 10^4$  cells/well in 24-well tissue culture plates. D-luciferin (Synchem, Kassel, Germany) at a concentration of 2 mg/ml was added immediately prior to the assay. Bioluminescence signal was measured using a plate reader (Berthold Tech., Bad Wildbad, Germany) and analyzed by Mikro Win 2000 software.

## **7.9 Immunocytostaining**

Cells ( $2 \times 10^6$ ) were seeded on coverslips placed in 24 wells and incubated for 24 h in DMEM (10% FBS and 1% P/S). The following day, cells were washed with PBS (3x5 min), fixed with paraformaldehyd (4%, 20 min) and washed with PBS (3x5 min). Cells were permeabilized with 0.2% Triton X-100 in PBS (3 min) and washed with PBS (3x5 min). Unspecific binding of antibodies was prevented by blocking the cells with 3% BSA for 60 min. Thereafter, cells were washed with 0.3% BSA/PBS and incubated for 90 min with the first antibody (in 0.3% BSA/PBS). Afterwards, cells were washed and incubated for 1 h with the secondary antibody (in 0.3% BSA/PBS) and washed again (PBS x 5, bidest x 1). Cover slips were removed from the 24 wells put on glass slides and sealed using Citi Fluor (Citifluor Ltd, England).

## **7.10 Flow cytometry analysis**

Cells were plated into 6 cm plates at  $1 \times 10^6$  cells per plate. Cells were treated with different concentrations of BCNU. At 24h, cells were trypsinized, combined with any floating cells, pelleted, washed twice with PBS, repelleted. All centrifugations were at  $700 \times g$  for 5 min at 4 C. Subsequently, cells were fixed in ethanol (final concentration, 70%) and stored at -20 C. Cells were pelleted and washed once with PBS. Cells were resuspended in 0.5 ml PBS with RNase (10 mg/ ml) and incubated for 30min at 37C. Cells were processed for propidium iodide (PI) staining (1mg/ml). Flow cytometric analysis was performed on FACs calibur from BD Biosciences. Cell cycle profiles were analyzed using Cell Quest software (BD FACSCalibur system).

### **7.11 Caspase-3/7 Assay**

The activities of caspase-3/7 were measured using the Caspase-Glo 3/7 kit ( Promega Corp., Madison, WI). Briefly, cells were grown in 96-well plates until the semiconfluency stage and then treated with indicated concentrations of BCNU for 24h in culture medium. Plates were removed from the incubator and kept at room temperature for 30 min and the Caspase-Glo 3/7 reagent (100  $\mu$ l) was added. Plates were further incubated on a shaker at room temperature for 2h and read using a luminometer.

### **7.12 Animal experiments**

All animal procedures were performed in accordance with the German laws for animal protection and were approved by the local animal care committee and the Bezirksregierung Köln. Three equivalent sets of animals (n =18 in total) were studied. Each set of animals (n=6) bearing test (n=2), negative control (n=2), and positive control (n=2) tumor cells was divided into non-treated (n=3) and BCNU-treated animals (n=3). The animals were under anesthesia by i.p. injection of ketanest (80 mg/kg) and rompun (16mg/kg). Tumor cells ( $1 \times 10^6$  cells in 50  $\mu$ l of DMEM) were injected subcutaneously into each site in the shoulders and flanks of nude mice (approximately 30g body weight) as follows: U87dEGFR-E2F-TA-LITG (test, n=2); U87dEGFR-TA-LITG (negative control, n=2) and U87dEGFR-LITG (positive control) giving rise to 4 tumors per animal. On days 7 to 9 post tumor implantation, xenografts reached a size of ~5 mm in diameter, and BCNU (40 mg/kg body weight) was injected (i.p.) in 50 % of animals.

### **7.13 Bioluminescence imaging *in culture and in vivo***

For the assessment of cells in culture, D-luciferin (Synchem, Kassel, Germany) was added to tissue culture medium to a final concentration of 2 mg/ml. Five minutes later, photons were counted using the IVIS200 imaging system (Xenogen, Alameda, CA) according to the manufacturer's instructions. Data were analyzed using LivingImage software (version 2.50, Xenogen). For *in vivo* studies, mice were injected with 200µl of the D-luciferin (20mg/ml) solution intraperitoneally. 10 minutes after D-luciferin injection, each mice was placed in the specimen chamber mounted with a CCD camera cooled to – 120°C with a field of view (FOV) set at 12.8 cm above the sample shelf. The photon emission transmitted from cell samples and mice were measured with standard image acquisition times ranging between 1-300 seconds. The gray scale photographic images and bioluminescence pseudocolor images were superimposed using the LivingImage V

2.50 software overlay (Xenogen). A region of interest (ROI) was performed to quantify different signal intensities.

### **7. 14 Statistics**

Descriptive statistics and regression analysis were performed with Microsoft Excel 2002 (Microsoft Corp). Student's t-test as well as Mann-Whitney Rank sum test were performed with SigmaStat 3.0 (SPSS Inc.); statistical significance was set at the less than 5% level ( $p < 0.05$ ).

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## 9. Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit -einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie- abgesehen von unten angegebenen Teilpublikationen -noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Sigrun Korsching betreut worden.

Köln, April 2007

Parisa Monfared

### Teilpublikationen im Rahmen dieser Arbeit:

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## 10. Lebenslauf

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