# IDENTIFICATION OF SIGNALLING PATHWAYS REGULATING TBX2 GENE EXPRESSION AND ITS TARGET GENES

**by**

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**TO**

**MY MOTHER AND FATHER**

**MY WIFE AND DAUGHTER**

# CONTENTS

## ABSTRACT

**Identification of signalling pathways regulating TBX2 gene expression and its target genes**

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Members of the T-box family of transcription factors provide an important link between development and cancer. T-box factors play critical roles in embryonic development and results from recent studies suggest that they function in controlling cell cycle progression and also in the genesis of cancer. Importantly, the T-box factors Tbx2 and Tbx3 are overexpressed in several cancers including melanoma, small cell lung carcinoma, breast, pancreatic, liver and bladder cancers and can suppress senescence, a cellular process which serves as a barrier to cancer development. However, the precise role of most T-box factors is poorly defined, in part, because their target genes are still poorly characterised and very little is known of the signalling pathways that regulate their expression and activity.

The broad aim of this study was therefore to contribute towards the identification of Tbx2 target genes as well as to identify signalling pathways that regulate TBX2 expression. The specific aims were thus to (1) investigate the regulation of type1 collagen gene expression by Tbx2; (2) clone the human TBX2 regulatory region and to identify cis-acting elements involved in the basal transcription of the TBX2 gene and (3) investigate the regulation of TBX2 gene expression by signalling pathways.

Microarray analysis have previously implicated the collagen I genes as potential Tbx2 targets. To further investigate this possibility, this study firstly examined the expression of TBX2 in several human fibroblast cell lines and show that it while it is expressed in normal fibroblasts, it drastically reduced in its transformed counterparts. This pattern of TBX2 expression correlated with that observed for the human α2(1) collagen gene (*COL1A2*). Interestingly, stable expression of transfected TBX2 in transformed fibroblast cell lines further reduced expression of the human endogenous *COL1A2* gene. This ability of Tbx2 to repress the human *COL1A2* gene was confirmed in luciferase reporter assays and shown to be independent of the consensus T-box binding element.

To better understand the molecular mechanisms regulating TBX2 gene expression, the 5'-flanking region of the human TBX2 gene was isolated and characterized. The proximal promoter (-216/+32) was shown to mediate most of the basal activity and a proximal Sp1 site juxtaposed to an inverted CCAAT box element were both shown to be essential for basal promoter activity. While a physical and functional interaction between Sp1 and NF-Y was not essential for optimal basal promoter activity, a dynamic balance in their levels appears to be important. Furthermore, the data reveal a potential downstream promoter element in the TBX2 promoter which significantly influences basal activity.

Finally, this study explored the regulation of TBX2 expression by signalling pathways and demonstrated that 12-O-tetradecanoylphorbol-13-acetate (TPA) induces TBX2 expression in normal human fibroblasts in a Protein Kinase C (PKC)-dependent and Mitogen Activated Protein Kinase (MAPK) independent manner. However, the MAPK signalling pathways were implicated in regulating TBX2 gene expression in transformed human fibroblasts. The data further revealed that TPA activates transcription of TBX2 through histone H3 phosphorylation, and thereby recruiting Sp1 to the TBX2 gene. In addition, TPA activated mitogen- and stress-activated protein kinase 1 (MSK1) which correlated with phosphorylation of histone H3 in a PKC-dependent and MAPK-independent manner. This study is the first to provide evidence that phosphorylation of histone H3 leads to the transcriptional activation of the TBX2 gene and to link MSK1 to the PKC pathway. It thus provides an important insight into signalling pathways that regulate TBX2 gene expression.

# CHAPTER 1:

# INTRODUCTION

## 1.1. T-box gene family

The T-box family of transcription factors plays a central role in embryonic development and is an important aspect of developmental biology. T-box genes encode a family of transcription factors that are characterized by a conserved DNA binding domain called the T-box. Members of the T-box gene family have been identified in a wide variety of animal species ranging from Hydra to humans and play remarkably important roles in embryonic development such as the specification of primary germ layers and cell fates during organogenesis (Papaioannou, 2001; Showell et al, 2004; Naiche et al, 2005). Their important regulatory roles in development have been demonstrated by mutational studies where mutant alleles, including heterozygotes, commonly give a phenotype. For example, the prototype of the family, the T-gene (Herrmann et al., 1990) encodes the Brachyury transcription factor (Kispert et al., 1995), and mutations within this gene affect mesoderm induction, while mutations in the human Tbx3 and Tbx5 genes result in the autosomal dominant Ulnar-Mammary (Bamshad et al., 1997) and Holt-Oram (Li et al., 1997) syndromes respectively. Both syndromes are characterized by developmental defects; in Ulnar-Mammary syndrome the limb and apocrine glands are affected, while Holt-Oram syndrome is characterized by abnormalities in the cardiac septum and forelimbs. Dosage sensitivity or haploinsufficiency is postulated to be the mechanism by which mutations in T-box genes are responsible for these developmental defects. In addition to naturally occurring mutations, targeted disruption of the mouse Tbx6 gene results in an embryo in which the somites are transformed into neural tubes (Chapman and Papaioannou, 1998) while disruption of the Tbx4 and Tbx5 genes affect limb identity (Rodrigues-Estaban et al., 1999; Takeuchi et al., 1999).

The prototype T-box family member, Brachyury (or T for Tail) has long been known to play an important role in mouse development, and was cloned from mouse by Herrmann et al. in 1990. Brachyury was first identified to be a member of a protein family when a study in Drosophila melanogaster showed that the DNA binding sequence of the *omb* gene is highly homologous with that of Brachyury (Pflugfelder et al., 1992). In 1994, work by Bollag et al revealed that a T-box family of transcription factors exists. Since then, a

number of T-box members have been identified that share the conserved DNA binding domain. To date, 19 T-box members have been characterized in vertebrates and, based on phylogenetic analysis, the T-box gene family was shown to fall into several subfamilies (Fig. 1.1.) (Naiche et al., 2005).

T-box proteins are DNA-binding transcription factors because they recognize and bind specific DNA sequences to regulate their target gene expression. Sequence characterization to identify a Brachyury binding motif revealed a palindromic sequence (TTT(G/C)ACACCTAGGTGTGAAA) to which Brachyury binds as a monomer or dimer (Kispert et al., 1993). To date, all members characterised have been shown to bind the core sequence GGTGTGA, referred to as the T-element, as monomers (Wilsion and Conlon, 2002). Some members have, however, been shown to recognize several non-canonical sequences (Ghosh et al., 2001; Carreira et al., 1998; Lingbeek et al., 2002). Although all T-box proteins recognize similar DNA sequences, several lines of evidence indicate that they regulate different target genes which may depend on the promoter context and cofactors (Conlon et al., 2001; Muller et al., 1997; Coll et al., 2002).

As transcription factors, T-box proteins have been shown to either activate or repress gene expression and transcriptional regulatory domains have been mapped in several T-box proteins (Kispert et al., 1995; Stennard et al., 2003; Zaragoza et al., 2004). Recent studies have shed light on some of the mechanisms by which T-box factors regulate their target genes. For example, Tbx19 was shown to activate transcription by recruiting SRC/p160 coactivators to its cognate DNA target in the pituitary pro-opiomelanocortin gene promoter (Maira et al., 2003). Tbr1 on the other hand forms a complex with the nucleosome assembly proteins CASK and CINAP to regulate expression of genes such as *NR2b* and *reelin* (Wang et al., 2004). Interestingly, a recent study revealed that interaction of the Xenopus T-domain protein Xbra with Smad1, an effector of BMP signalling, prevents it from inducing expression of *goosecoid* (Messenger et al., 2005). Despite these contributions, there is still a paucity of information regarding the molecular mechanism(s) involved in the transcriptional regulation by T-box factors.

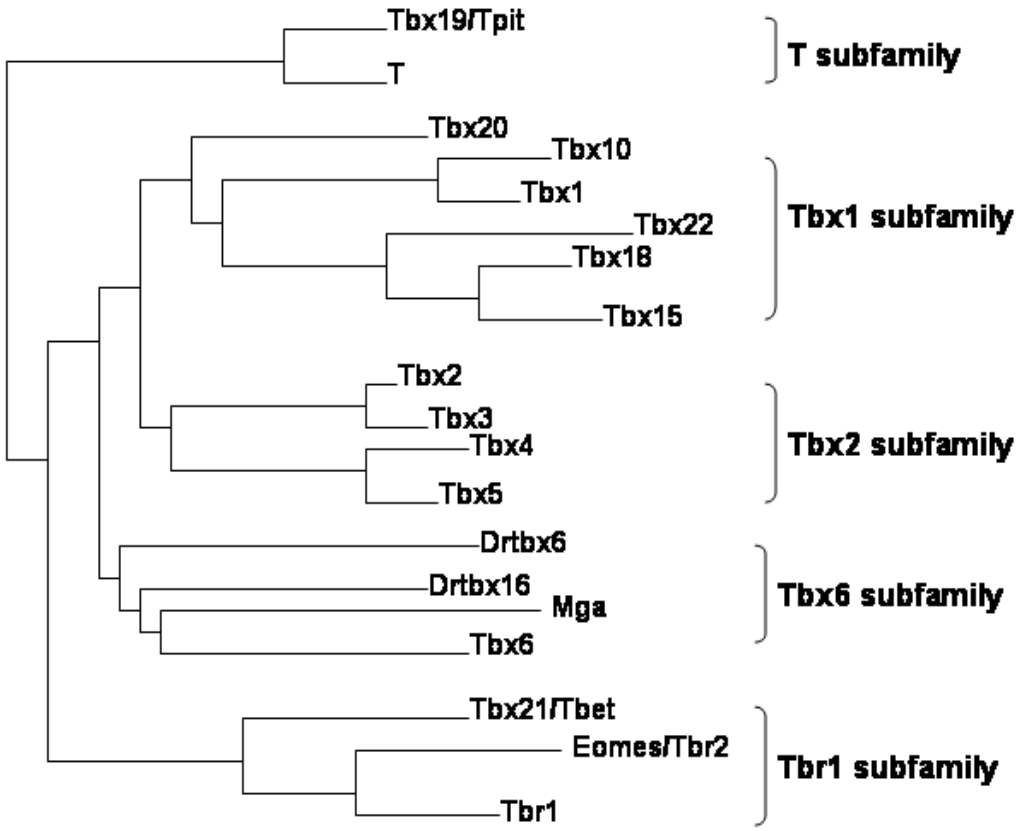



**Figure 1.1.** Schematic phylogenetic tree of the T-box gene family of vertebrates, showing the relationship of genes in the five subfamilies indicated by brackets on the right. All of these genes are present in mammals with the exception of the zebrafish genes Drtbx6 and Drtbx16, which do not have orthologs in mammals (from Naiche et al., 2005).

This review will provide a general overview of the current literature available on the regulation of Tbx2 gene expression and its target genes, and will introduce key areas of research pertaining to this thesis.

## 1.2. Tbx2

In mice, *Tbx2* is expressed in adult heart, lung, kidney, ovary and in cells of the melanocyte lineage (Bollag et al., 1994; Carreira et al., 1998) and in humans it is expressed in a wide variety of tissues including fetal kidney and lung as well as in a number of adult tissues such as kidney, lung, placenta, ovary, prostate, spleen, testis, breast, heart, thymus, intestine and polymorphonucleocytes (Law et al., 1995; Campbell et al., 1995). Human Tbx2 has not yet been linked to any known genetic syndrome but has been implicated in limb, heart and mammary gland development (King et al., 2006; Plageman and Yutzey, 2005; Rowley et al., 2004). Targeted mutagenesis to investigate Tbx2 function in mice have shown that heterozygous mutants appear normal while homozygous mutants die between 10.5 and 14.5 days post coitum of cardiac insufficiency (Plageman and Yutzey, 2005). This result suggests a crucial role for Tbx2 during cardiac development.

In addition to its key function in development, evidence is accumulating to suggest a role for Tbx2 in cancer. For example, Tbx2 and the very closely related factor Tbx3, have been implicated in cell cycle regulation and their expression is upregulated in a number of cancers, including breast (Sinclair et al., 2002; Fan et al., 2004), pancreatic (Mahlamäki et al., 2002; Hansel et al., 2004), melanoma (Vance et al., 2005; Hoek et al., 2004), liver (Renard et al., 2007) and bladder (Ito et al., 2005). Both Tbx2 and Tbx3 function as transcriptional repressors (Carreira et al., 1998; Carlson et al., 2001; He et al., 1999; Sinha et al., 2000), and have been shown to prevent senescence through a mechanism involving their ability to repress the cyclin dependent kinase inhibitors (Cdkis) $p19^{ARF}$ (Jacobs et al., 2000; Brummelkamp et al., 2002; Lingbeek et al., 2002) and $p21^{WAF1/CIP1/SDII}$ (referred to as p21) (Prince et al., 2004; Vance et al., 2005).

*Tbx2* encodes a transcriptional repressor and belongs to the Tbx2 subfamily which also includes *Tbx3, Tbx4* and *Tbx5*. In mouse, *Tbx2* is located within chromosome 11 (Bollag et al., 1994) and in humans, TBX2 has been mapped to chromosome 17q23 (Campbell et al., 1995). The T-box region of the human TBX2 transcription factor shares 90% DNA sequence homology and 96% peptide sequence homology with its mouse counterpart

(Law et al., 1995). Both human and mouse Tbx2, which span 3.378 kb and 2.281 kb respectively, possess 7 exons

The human Tbx2 protein consists of 702 amino acids with the T-box DNA-binding domain located at amino acids 104-285, whereas in mouse the T-box is located at amino acids 114-295 within the 711 amino acid protein. Due to the high degree of homology between the DNA-binding domain of Brachyury and Tbx2, investigators have utilised Brachyury as a model to gain insight into the DNA-binding specificity of Tbx2. These studies show that Tbx2 binds as a monomer to the Brachyury single half-sites (Carreira et al., 1998) and that it binds both the Brachyury palindromic and half sites equally well (Sinha et al., 2000). Mutating an arginine at position 122, which is conserved in all T-box factors, abolished the DNA-binding activity of Tbx2 in vitro without affecting its stability and nuclear localisation (Sinha et al., 2000).

Despite the potentially important role that Tbx2 plays in development and carcinogenesis as desribed above, the precise molecular function of Tbx2 is still relatively poorly understood. An improved understanding of this role of Tbx2 would require the elucidation of how Tbx2 gene expression is regulated, as well as the identification of potential Tbx2 target genes. The next section provides an overview of the, limited, studies carried out to date in this regard.

### 1.2.1. Tbx2 and its target genes

Tbx2 functions to control cell differentiation, proliferation and fate through transcriptionally repressing its target genes. *Tbx2* expression has been observed in several melanocyte and melanoma cell lines but not in pre-melanoblast cells (Carreira et al., 1998). Melanoblasts are neural crest derived cells which migrate to the hair follicles and epidermis where they differentiate into mature melanocytes capable of producing pigment (Silvers, 1979). While Tbx2 was first identified to repress the promoter of the melanocyte-specific tyrosinase-related protein 1 by binding the consensus T-box motif GTGTGA, it was also shown to bind the non-canonical GTGTTA and GGGTGA sequences in vitro (Carreira et al., 1998).

Recent studies have identified several Tbx2 target genes that play a role during early heart development. The formation of the chamber and non-chamber myocardium

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

During heart development the non-chamber myocardium retains the embryonic myocardial phenotype of the tubular heart longer than the chamber myocardium. The Nppa gene is specifically expressed in the developing chamber myocardium and is one of the first hallmarks of chamber formation (Christoffels et al., 2000), which raises the question of how this gene is repressed in the non-chamber myocardium. This was addressed by studies which found that mutating two adjacent binding sites for T-box factors and Nkx2.5, respectively, abrogated repression of *Nppa* in regions of the non-chamber myocardium (Habets et al., 2002). Furthermore, Tbx2 was shown to co-operate with Nkx2.5 to suppress *Nppa* promoter activity in embryonic myocardium (Habets et al., 2002). Interestingly, the physical and functional interaction between Nkx2.5 and Tbx5 were shown to be involved in synergistically activating an *Nppa*-reporter gene (Bruneau et al., 2001; Hiroi et al., 2001). Moreover, Tbx2 was shown to decrease Nkx2.5-Tbx5-mediated activation of the *Nppa* promoter. (Habets et al., 2002) Taken together,

researchers believe that Nppa expression is regulated by the competition between Tbx2 and Tbx5 to bind and cooperate with Nkx2.5.

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

Type 1 collagen synthesis is crucial for normal embryonic development and in maintaining tissue integrity, and its aberrant expression has deleterious effects on several biological processes including bone development (Bornstein and Sage, 1989). In an attempt to identify genes that may be regulated by Tbx2, cDNA microarray analysis was performed on mouse NIH 3T3 fibroblasts overexpressing Tbx2 and results revealed that the type 1 collagen gene was upregulated (Chen et al., 2001). Interestingly, a parallel investigation in which *Tbx2* was overexpressed in the rat ROS17/2.8 osteoblastic cell line showed downregulation of type 1 collagen (Chen et al., 2001). Taken together, these contrasting results suggest that Tbx2 may regulate type 1

collagen genes by functioning as either a co-activator or a co-repressor depending on the cell context and/or the species.

A possible mechanism for how Tbx2 may contribute towards the oncogenic process is suggested by studies which have shown that it is able to function as an immortalizing gene that enables cells to bypass senescence (Jacobs et al., 2000;). The main mediators of senescence are the Cdkis, p21 and $p16^{INK4a}$, with p21 thought to be necessary for initiating the senescence-like growth arrest and $p16^{INK4a}$ required for maintenance of the state (Stein et al., 1999). In an attempt to investigate the potential mechanism by which Tbx2 causes mouse embryo fibroblasts to bypass senescence, $p19^{ARF}$ was revealed as a candidate target gene of Tbx2 (Jacobs et al., 2000). Subsequent work showed that the $p14^{ARF}$ (human homolog of $p19^{ARF}$) promoter contains a functional Tbx2 binding motif (Lingbeek et al., 2002). Tbx2 was also shown to directly repress $p21^{WAF}$ through binding a putative T-element in its promoter (Prince et al., 2004). Interestingly, loss of Tbx2 function in mouse embryos has no effect on the expression of $p21^{WAF}$ and $p19^{ARF}$ (Harrelson et al., 2004) which suggests that another factor, such as a closely related T-box member, may substitute for Tbx2 in the cell cycle. The possibility that Tbx2 may contribute to the oncogenic process by inhibiting senescence is supported by a study that showed that expressing a dominant-negative Tbx2 in B16 mouse melanoma cells led to increased levels of p21 and induction of senescence (Vance et al., 2005).

### 1.2.2. Regulation of Tbx2 gene expression

Given the critical role that Tbx2 plays in embryonic development and its implication in carcinogenesis it is important to understand the signalling pathways which regulate its gene expression. The role of Tbx2 seems to be tightly linked to the activity of the Bone morphogenic proteins (BMPs), Sonic hedgehog *(*Shh), and Wnt signalling pathways and a few examples supporting this are discussed below.

BMPs belong to the TGF-β family of signalling transducers and are important in the development of the non-chamber myocardium (Délot, 2003). There is accumulating evidence that both *Tbx2* and *Tbx3* may be downstream Bmp2 targets during heart development. Firstly, the expression patterns of *Tbx2* and *Tbx3* overlap significantly with *bmp2* during chick heart development (Yamada et al., 2000). Secondly, ectopic

expression of *Bmp2* was shown to induce *Tbx2* and, to a lesser extent, *Tbx3* expression in non-cardiogenic tissue that is competent to form cardiac tissue. Moreover, in the same study when chick cardiac tissue explants were incubated with Noggin, a panantagonist of BMP signalling, *Tbx2* was downregulated with only very little effect on *Tbx3*. Furthermore, mouse *bmp2*$^{-/-}$ embryos show marked morphological abnormalities in cardiac development (Zhang and Bradley, 1996) and *Tbx2* gene activity is substantially reduced in the cardiac region of these embryos (Yamada et al., 2000).

The hypothalamus is situated in the area of the brain just below the thalamus and is important in regulating body temperature, blood pressure, weight regulation, and many other autonomic-nervous system activities such as fear, rage and sexual behaviors (van de Graaff, 2002). Both in vitro and in vivo studies have implicated Tbx2 in the patterning and development of the hypothalamus. Much of the evidence for this results from work done in chick on a subset of hypothalamic cells, termed the ventral tubero-mamillary (vt-m) cells which arise from a set of floor plate-like precursors (Manning et al., 2006). The floor plate-like precursors initially express Shh but require its downregulation in order to progress to vt-m cells. BMPs have previously been shown to downregulate *Shh* in a wide variety of tissues and Manning et al. (2006) show that downregulation of Shh in the vt-m cells occurs by a BMP-Tbx2 signalling pathway. Both overexpression and knock-down experiments showed that Tbx2 downregulates Shh in the vt-m hypothalamus. Furthermore, Jeong and Epstein (2003) identified a T-element in the mouse Shh promoter and they show that mutating this T-element results in aberrant expression of Shh within the hypothalamus. In Manning et al. (2006), BMPs were shown to upregulate Tbx2, which may involve the ability of BMPs to antagonise Wnt. In the presence of the BMP inhibitor, chordin, expression of Tbx2 was downregulated in the vt-m hypothalamus. In contrast, blocking BMP and Wnt signalling was shown to rescue the expression of *Tbx2* in the vt-m cells. The results by Manning et al. (2006) raise the possibility that the T-element in Shh is regulated by Tbx2 in the hypothalamus.

While several signalling pathways, including those mentioned above, have been associated with the regulation of *Tbx2* expression, the details of this regulation remain largely unknown. Furthermore, key cis-acting elements responsible for Tbx2 gene expression have not yet been identified, and relatively few studies have reported on the molecular mechanism(s) regulating Tbx2 expression. The 5'-flanking region of the

mouse *Tbx2* gene has been cloned and the transcription factor Mitf was shown to activate *Tbx2* gene expression by recognizing an E-box element in melanocytes (Carreira et al., 2000). A putative retinoic acid response element was also identified in this promoter and it was shown to be responsible for retinoic acid stimulated *Tbx2* gene expression (Boskovic and Niles, 2004). Both Tbx2 and Tbx20 play a role in heart development and a recent study showed that Tbx20 was able to bind in vivo to a 5'-regulatory region of the mouse *Tbx2* gene, which contains a putative T-element. This finding suggested that *Tbx2* is a direct target for repression by Tbx20 in the developing heart (Cai et al., 2005).

A major aspect of the current thesis was to clone the 5'- regulatory region of the human *Tbx2* and to identify cis-acting elements involved in its expression and hence the next section will provide an overview of the areas relevant to this study.



## 1.3. Cis-acting elements in the proximal promoters of eukaryotic genes

Transcriptional control is the key means of regulating gene expression. In eukaryotes, transcription of protein-coding genes is carried out by RNA polymerase II, a multisubunit complex. RNA polymerase II requires a number of general transcription factors to recognize the transcription start site and forms a stable transcription-initiation complex with these factors. The core promoter is identified as a DNA sequence to which RNA polymerase II binds directly and which determines the site of transcription initiation. Typically, the core promoter comprises DNA sequences within -40 to +40 nucleotides relative to the transcriptional start site. In addition to the core promoter, the proximal promoter (approximately 200 bp) generally contains multiple recognition sites for sequence specific-binding transcription factors (Blackwood and Kadonaga, 1998; Butler and Kadonaga, 2002). A schematic diagram of cis-acting elements typically found in a proximal promoter is depicted in Fig 1.2.

### 1.3.1. Core promoter elements

Several types of core promoter elements have been well characterized, including the TATA box, Initiator (Inr) and the downstream promoter element (DPE).

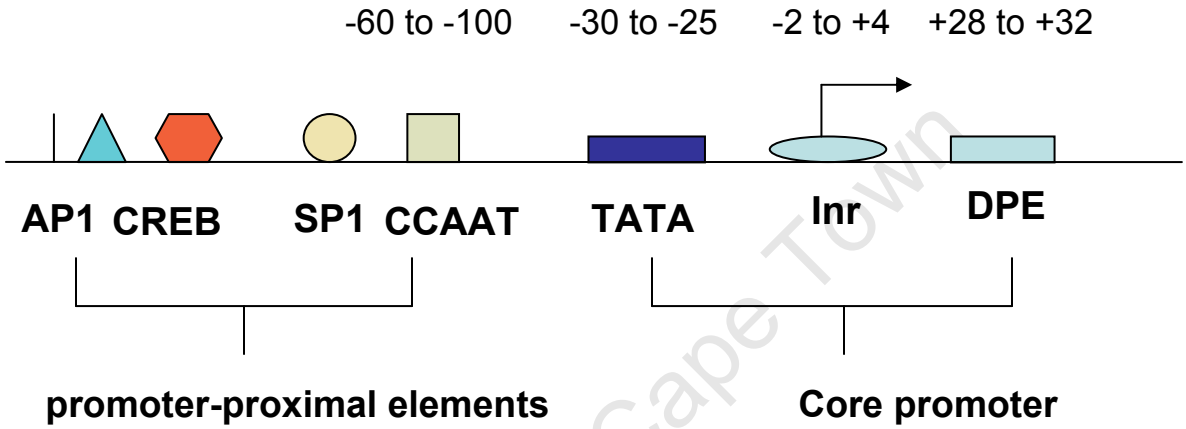



**Figure 1.2.** Structure of the proximal promoter of a typical eukaryotic protein-coding gene. Some potential regulatory elements are depicted. The core promoter includes the TATA box, Initiator (Inr) and downstream promoter element (DPE). Usually, only one or two of these core promoter elements are found in a specific gene. Some genes contain none of these elements. Immediately upstream of the core promoter (from -50 to approximately -200 relative to start site), there are typically multiple elements for sequence-specific DNA-binding transcription factors. Sp1 and CCAAT box elements are the most common promoter-proximal elements.

### 1.3.1.1. The TATA box

The TATA box was the first eukaryotic core promoter element to be identified (Breathnach and Chambon, 1981). The TATA box is an A/T-rich sequence, typically located from -25 to -30 bp relative to the transcription start site. The consensus sequence for the TATA box is TATAAA. Various non-canonical sequences have, however, also been reported that can function as a TATA box (Singer et al., 1990). A statistical analysis of 1031 human core promoters indicated that approximately 32% have a potential TATA-box motif (Suzuki et al., 2001). The TATA-box is bound by the TATA box-protein (TBP), which is a key component of transcription factor IID (TFIID) which is a general transcription factor.

### 1.3.1.2. The Inr element

The Inr element was defined as a core promoter element of discrete function. It encompasses the transcription start site, and its consensus sequence in mammals is Py-Py A (+1) N T/A Py Py (Bucher, 1990; Corden et al., 1980). Although the Inr can function independently, it was often found to cooperate with other core promoter elements and has been shown to be recognized by TFIID (Burke and Kadonaga, 2006).

### 1.3.1.3. The DPE

The DPE is most commonly found in TATA-less promoters and it seems likely that it is a compensatory element for the TATA box. The DPE is generally found at a downstream position (+28 to+34) and its consensus sequence is A/G-G-A/T-C/T-G/A/C. A functional Inr element is often found in the DPE-dependent promoters. Although the DPE has been shown to be present in human genes, most work reporting on its involvement in transcriptional regulation has focused on Drosophila genes (Smale and Kadonaga, 2005; Kadonaga, 2002). The role of the DPE in the regulation of human genes is therefore poorly understood.

### 1.3.2. Promoter-proximal elements

In addition to the elements found in the core promoter, DNA motifs for sequence-specific DNA binding transcription factors are also needed for efficient transcription of a specific gene. These elements reside in the proximal promoter and are typically located -40 to -200 bp from the transcription start site. The Sp1 and CCAAT box motifs will be discussed in more detail in this review because they are the most common promoter-proximal elements and are of particular importance to this study.

#### 1.3.2.1. The CCAAT box

The CCAAT box is one of the most common cis-acting elements present in the promoter of eukaryotic genes. A statistical analysis of eukaryotic genes revealed that the CCAAT box element is present in 30% of the 502 promoters analysed (Bucher et al., 1990). Typically, the CCAAT box is found as a single copy element in either the forward or reverse orientation from -60 to -100 bp relative to the transcriptional start site. As a critical cis-acting element, the CCAAT box motif has been shown to be essential for basal transcription of many genes. The extent of dependence on the CCAAT box motif is variable and depends on the context of promoters. In relatively simple TATA box-less promoters containing only one or two elements, the CCAAT box is absolutely necessary for gene transcriptional activation. In contrast, the CCAAT box motif is somewhat less important for some TATA box-containing promoters. Although several nuclear factors have been described to bind the CCAAT box motif, it is now well-documented that NF-Y is the major binding factor.

NF-Y is a transcription factor complex that consists of three subunits, NF-YA, NF-YB and NF-YC, all of which are required for CCAAT box binding. NF-Y is highly conserved among species, and is ubiquitiously expressed. The consensus sequence for NF-Y is C G/A G/A CCAAT C/G A/G C A/C (Mantovani, 1998; Mantovani, 1999; Maity et al., 1998). A tight association between NF-YB and NF-YC is required for NF-YA binding and further binding of the three subunits to the CCAAT box. NF-Y has also been shown to associate with TATA box binding protein (TBP) (Bellorini et al., 1997) and several TBP-associated factors (TAFs) (Frontini et al., 2002). In a recent study, Kabe et al. (2005) have shown that NF-Y is essential for the recruitment of RNA polymerase II in several CCAAT box-

containing genes. These findings are consistent with the important role of the CCAAT box element in gene transcription.

### 1.3.2.2. The Sp1 element

The Sp1 element is a G/C-rich motif and was initially identified as the specific binding element for the transcription factor Sp1. Sp1 sites are functional in both forward and reverse orientation with the consensus sequence being G(T)GGGCGG(G/A) (G/A)(C/T) (Kadonaga et al., 1986). A large number of genes have been shown to contain one or more Sp1 sites and they play a particularly critical role in maintaining gene expression in genes that lack a TATA-box. As is the case for NF-Y, Sp1 was also shown to associate with TBP and several TAFs (Emili et al., 1994; Chiang et al., 1995; Tanese et al., 1996). Although a single Sp1 site has been shown to be sufficient for Sp1 transcriptional activation, multiple Sp1 sites or additional cis-acting elements are required for efficient Sp1 transactivation depending on the promoter. For example, physical and functional interaction between the Sp1 and NF-Y transcription factors were previously reported to be involved in transcriptional activation of several genes (Liang et al., 2001; Yamada et al., 2000).

## 1.4. Sp1 and the regulation of gene transcription

Sp1 was one of the first eukaryotic transcription factors to be identified and cloned (Dynan and Tjian, 1983; Kadonaga et al., 1987). It has been shown to regulate a large number of genes by recognizing and binding GC-rich motifs. To date, it is well-known that Sp1 is a member of the Sp/XKLF family of transcription factors, and all members bind similar GC-rich sequences. A subfamily of Sp factors includes Sp1-Sp8, of which Sp1, Sp2, Sp3 and Sp4 contain similar structural modules. Unlike the rest of this subfamily, Sp1 and Sp3 are ubiquitously expressed (Bouwman et al., 2002).

As a sequence-specific binding transcription factor, Sp1 has a DNA-binding domain that contains three contiguous Zinc fingers and four discrete functional domains that are responsible for transcriptional activation (See Fig. 1.3.). Activation domains A and B are glutamine rich, and each are flanked at their 5' ends by a serine/threonine-rich region and the C domain is highly charged (Courey and Tjian, 1988; Philipsen and Suske,

1999). In addition to playing a critical role in maintaining target gene expression, Sp1 also exerts an important role in regulating gene expression (Samson and Wong, 2002).

### 1.4.1. Sp1 cooperation with factors involved in transcriptional regulation

Sp1 has been shown to bind a growing list of proteins involved in transcriptional regulation which suggests that it has diverse functions in gene regulation. These proteins include sequence-specific transcription factors as well as coactivators and corepressors which are discussed below.

#### 1.4.1.1. Sp1 interacts with sequence-specific transcription factors

In addition to the CCAAT box binding protein NF-Y, a physical and functional co-operation was also found in vitro between Sp1 and other proteins such as Ets1, USF and E2F1 (Gegonne et al., 1993; Lin et al., 1996; Ge et al., 2003 ). However, whether these complexes regulate specific genes in vivo remains to be elucidated.

There is also evidence that Sp1 cooperates with Smads to induce the transcription of the Cdki $p15^{ink4B}$ in response to transforming growth factor-β (TGFβ). In this study, both the Sp1 site and the Smad-binding motif in the $p15^{ink4B}$ promoter were shown to be required for transactivation. Here, Smad3/4 directly associated with Sp1 which led to enhanced binding and transactivation of the $p15^{ink4B}$ promoter (Feng et al., 2000). Consistent with this finding, a similar regulatory mechanism was revealed in which Smad-mediated TGFβ upregulates p21 gene expression (Pardali et al., 2000).

Interestingly, a previous report indicated that epidermal growth factor (EGF) induces expression of the human 12(S)-lipoxygenase gene in epidermoid carcinoma A431 cells by upregulating the activating protein 1 (AP1) component, c-Jun. However, no AP1 binding site was found in the promoter region responsive to EGF and further investigations revealed that Sp1 acts as an anchor protein to recruit c-Jun to the promoter (Chen and Chang, 2000). Recent work by the same group also revealed that 12-O-tetradecanoylphorbol-13-acetate (TPA) can enhance c-Jun/Sp1 interaction by Protein Phosphatase 2B-mediated dephosphorylation of c-Jun (Chen et al., 2007). Furthermore, interaction of v-Jun with Sp1/3 results in the inhibition of expression of the osteonectin gene in primary chick embryo fibroblasts (Chamboredon et al., 2003).

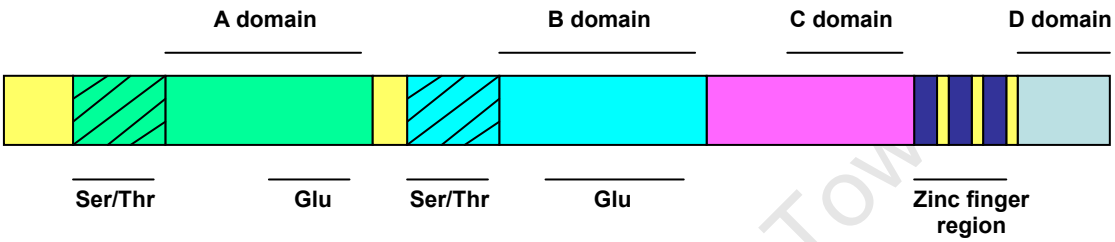



**Figure 1.3.** Structural features of the Sp1 protein. A, B, C and D represent the four transactivation domains and the zinc finger DNA-binding domain with three zinc fingers are shown in dark blue. Two serine and threonine rich regions are represented by Ser/Thr. Two glutamine rich regions are represented by Glu.

### 1.4.1.2. Sp1 interaction with coactivators and corepressors of transcription

In addition to interactions with sequence-specific transcription factors, Sp1 was also shown to exert an effect on transcription through interaction with transcriptional coactivators or corepressors. For example, the retinoblastoma protein (RB) was shown to directly associate with Sp1 in vivo, and to enhance transactivation of a Sp1-containing promoter (Udvadia et al., 1993). The coactivator p300 has also been shown to cooperate with Sp1 to regulate p21 promoter activity (Xiao et al., 2000). This was demonstrated to be due to the acetyltransferase region of p300 interacting with the DNA-binding domain of Sp1 and thereby increasing the DNA-binding activity of Sp1 (Suzuki et al., 2000). Moreover, ERK-mediated C-terminal phosphorylation of p300 enhances its association with Sp1 which is believed to be important for the regulation of EGF-induced keratin 16 gene expression (Chen et al., 2007).

Furthermore, Sp1 in association with either p107 or the nuclear orphan receptor EAR3 represses the Luteinizing hormone receptor (LHR) gene in a Sp1 site dependent manner. The latter result was obtained in a study that demonstrated that EAR3 associates with Sp1 at the LHR promoter and that this interferes with the interaction between Sp1 and the general transcription factor TFIIB (Zhang and Dufau, 2003). Phosphorylation of Sp1 however leads to its disassociation from p107 and the de-repression of the LHR gene promoter (Zhang et al., 2006).

### 1.4.2. Sp1 as a sequence-specific transcription factor

There is evidence that an increase in Sp1 protein levels in different cell systems leads to the upregulation in expression of several genes (Noe et al., 2001; D'Angelo et al., 1996; Pornttadavity et al., 2001; Sato and Furukawa, 2007). Current reports suggest that an interplay between Sp1 and other Sp proteins is involved in modulating cell-type-specific gene expression. Both Sp1 and Sp3 have similar affinities for GC-rich motifs and Sp3 can compete with Sp1 to bind to the same GC-rich elements. Thus it is not difficult to imagine that an alteration in the Sp1/Sp3 ratio may affect transcriptional regulation of target genes. Indeed, Sp3 has been shown to activate or repress transcription depending on cell type and/or promoters. Its ability to repress transcription results from it preventing Sp1 from binding its target DNA (Samson and Wong, 2002). For example, in human umbilical vein endothelial cells, hypoxia leads to the increase in expression of

cyclooxygenase-2 which is thought to be due to an increase in Sp1 protein. In this experiment, Sp3 protein levels remain unchanged (Xu et al., 2000). In contrast, interleukin-1β down-regulates type II collagen gene expression in articular chrondrocytes because of an increase of the Sp3/Sp1 ratio (Chadjichristos et al., 2003).

Similar to the interplay between Sp1 and Sp3, other transcription factors may also influence the ability of Sp1 to transactivate genes depending on the position of their binding sites. One example is Egr1 which has been shown to compete for binding to an overlapping motif in which both Sp1 and Egr1 can bind (Cao et al., 1993).

### 1.4.3. Phosphorylation of Sp1

Protein phosphorylation is one of the most common post-translational modifications involved in rapidly regulating transcription factor activity (Whitmarsh and Davis, 2000). Work by Jackson et al. in 1990 first revealed that Sp1 is phosphorylated by a DNA-dependent protein kinase, but this phosphorylation has not been shown to affect the activity of Sp1. In recent years, a growing body of evidence has indicated that Sp1 phosphorylation is not a constitutive modification but results from a variety of extracellular stimuli and a number of kinases, including PKC, PKA, DNA-PK, ERK, CK2 and JNK. Phosphorylation of Sp1 has been shown to regulate its transcriptional activity, resulting in both positive and negative regulation of the expression of specific genes (Chu and Ferro, 2005; Samson and Wong, 2002; Chu and Ferro, 2006).

Sp1 contains many potential phosphorylation sites with some being located in both its binding and transactivation domains. Thus, Sp1 can be phosphorylated at different sites or combinations of sites which may yield a different effect on Sp1 activity. Importantly, phorbol esters, well-known activators of PKC, up-regulate Sp1 mediated gene expression by phosphorylating Sp1 and consequently increasing its DNA binding activity in human HepG2 cells (Zheng et al., 2000). Similarly, atypical PKC-dependent phosphorylation of Sp1 leads to an increase in its DNA binding activity which is required for up-regulated platelet-derived growth factor B-chain gene expression (Rafty and Khachigian, 2001). ERK-mediated phosphorylation of Sp1 was also proposed to have an effect on its transcriptional activation of the endothelial growth factor gene in fibroblasts and Drosophila cells. The same investigators identified Thr453 and Thr739 as ERK target sites in the Sp1 protein (Milanini-Mongiat et al., 2002). In addition, PKA-dependent

phosphorylation of Sp1 was also shown to enhance Sp1 binding and transcriptional activity (Rohlff et al., 1997; Ahlgren et al., 1999).

In contrast to the above scenario, Casein Kinase 2 (CK 2) phosphorylates Sp1 to lead to a decrease of Sp1 DNA binding activity in rat liver and K562 cells. In this case, CK2 phosphorylates Sp1 on Thr579, which is in the second zinc finger in the DNA binding domain (Armstrong et al., 1997). Furthermore, loss of the reduced folate carrier (RFC) gene expression in antifolate-resistant leukemia cells is associated with an inhibitory Sp1 phosphorylation (Stark and Assaraf, 2006). Interestingly, when aortic smooth muscle cells were treated with fibroblast growth factor-2, Sp1 was phosphorylated by ERK1/2 which led to its increased DNA binding activity and repression of the platelet-derived growth factor receptor-alpha (Bonello and Khachigian, 2004).

Although phosphorylation frequently affects the binding activity of Sp1, some cases have been reported where Sp1 phosphorylation was shown to influence transcriptional activity without altering its DNA binding activity. For example, Hepatocyte growth factor-induced phosphorylation of Sp1 in keratinocytes enhances its ability to activate the VEGF promoter without affecting its binding activity (Reisinger et al., 2003). Similarly, in endothelial cells, shear stress mediates Sp1-dependent activation of the tissue factor promoter, but shear stress-induced Sp1 phosphorylation does not change Sp1 binding activity (Lin et al., 1997). One possible mechanism by which phosphorylation of Sp1 may affect its transcriptional activity in this case may be to regulate its interaction with other transcription proteins as was shown for p107 above (Zhang et al., 2006).

Protein phosphatases have also been shown to play an important role in Sp1 phosphorylation status and hence regulation of Sp1 activity. There is evidence that treatment with the phosphatase inhibitor Okadaic acid leads to an increase in Sp1 phosphorylation, and results in an increase or decrease Sp1 activity (Armstrong et al., 1997; Daniel et al., 1996; Vlach et al., 1995; Wang et al., 1999; Ray et al., 1999;). Consistent with this finding, in vitro studies showed that Sp1 dephosphorylation with protein phosphatases also regulate Sp1 DNA binding activity (Rohlff et al., 1997; Chupreta et al., 2000; Stark and Assaraf, 2006; Wang et al., 1999; Armstrong et al., 1997; Daniel et al., 1996).

Finally, Sp1 phosphorylation has also been associated with organ development and cell proliferation. High levels of phosphorylated Sp1 were found in the mature liver and lung as compared to the developing liver and lung respectively (Chu et al., 1999; Leggett et al., 1995). In NIH/3T3 cells, dephosphorylation of Sp1 was observed when cell growth was induced by serum (Lacroix et al., 2002). Levels of phosphorylated Sp1 were also observed to be dynamic throughout the cell cycle (Leggett et al., 1995; Black et al., 1999), and the cyclin-dependent kinase 2 (CDK2) has been suggested to be responsible for regulating this phosphorylation (Haidweger et al., 2001).

### 1.4.4. Chromatin structure and Sp1 activity

Transient modification of the chromatin structure is important in regulating transcription and a growing number of studies are now focussing on understanding this phenomenon. It is becoming clear that covalent modifications of histones play particularly critical roles in this process (discussed further in section 1.5.1). Compared to the various possible forms of histone modifications, acetylation is the best-understood. It is believed that acetylation results in reduced interaction between positively charged histone tails and negatively charged DNA, thus resulting in a relaxed chromatin structure which is more accessible to transcription factors. Deacetylation on the other hand is believed to result in chromatin condensation which restricts access of transcription factors to target DNA. Histone acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Grunstein, 1997; de Ruijter et al., 2003).

Interestingly, Sp1 also plays a role in transcriptional regulation by chromatin remodeling. In mouse, Sp1 recruits HDAC1 to Sp1 sites in the HDAC1 promoter with a resultant repression of HDAC1 expression. This role of HDAC1 in the repression of its own promoter is emphasized by the observation that in the presence of the deacetylase inhibitor trichostatin A (TSA) the same Sp1 sites are essential for activation of the HDAC1 promoter. These results suggest that there is an autoregulatory mechanism controlling *HDAC1* expression in mammalian cells (Schuettengruber et al., 2003; Doetzlhofer et al., 1999). Additional support for a negative role of histone acetylation in transcription was demonstrated in studies which showed that TSA induced human telomerase reverse transcriptase (hTERT) expression in normal cells, and activates the hTERT promoter in a Sp1 site dependent manner (Takakura et al., 2001). In similar

studies in Hela cells, HDAC inhibitors were also shown to activate *p21* transcription through a Sp1 site (Han et al., 2001). Finally, OX40, a member of the TNFR superfamily is expressed on activated T cells and regulates T cell-mediated immune responses. It was recently demonstrated that up-regulation of OX40 gene expression involves a mechanism of chromatin remodeling requiring a Sp1 site in the OX40 promoter (Tone et al., 2007).

As illustrated above, the role of Sp1 in regulating gene expression is complex and involves diverse mechanisms such as post-translational modification, protein-protein interaction, and alteration of chromatin structure.

## 1.5. Histone H3 phosphorylation and gene transcriptional activation

### 1.5.1. Structure and function of the nucleosome

In eukaryotic cells, the enormous length of genomic DNA must be packaged into a highly ordered and condensed structure called chromatin in order to fit within cells. The basic building unit of chromatin is the nucleosome which consists of a protein core with 146 base pairs of DNA wound around it (Fig. 1.4.). The protein core is an octamer containing two copies each of histones H2A, H2B, H3 and H4. These core histones have a similar structure with a basic N-terminal domain, a globular domain and a C-terminal tail. The histone-fold domains of the four core histones are responsible for histone-histone and histone-DNA interactions (Luger et al., 1997).

N-terminal tails of histones protrude outside of the nucleosomes and are subject to a variety of covalent post-translational modifications, including acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation. These modifications are generally transient and dynamic and result in alteration of chromatin configuration (Nowak and Corces, 2004) (Fig. 1.5.), which in turn may affect access of transcription factors to their target promoters and hence gene expression. For example, phosphorylation of the N-terminal tail of histone H3 has been linked to transcriptional activation of the c-fos and c-jun immediate-early (IE) response genes (Mahadevan et al., 1991). Phosphorylation of histone H3 on serine 10 was first observed in association with chromosomal condensation (Gurley et al., 1978) and is therefore discussed below.

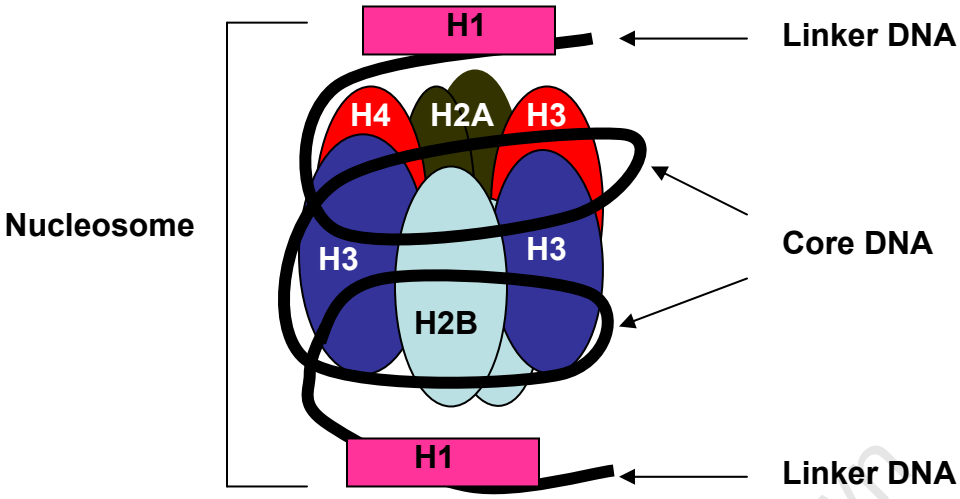



**Figure 1.4.** Schematic representation of the nucleosome structure.

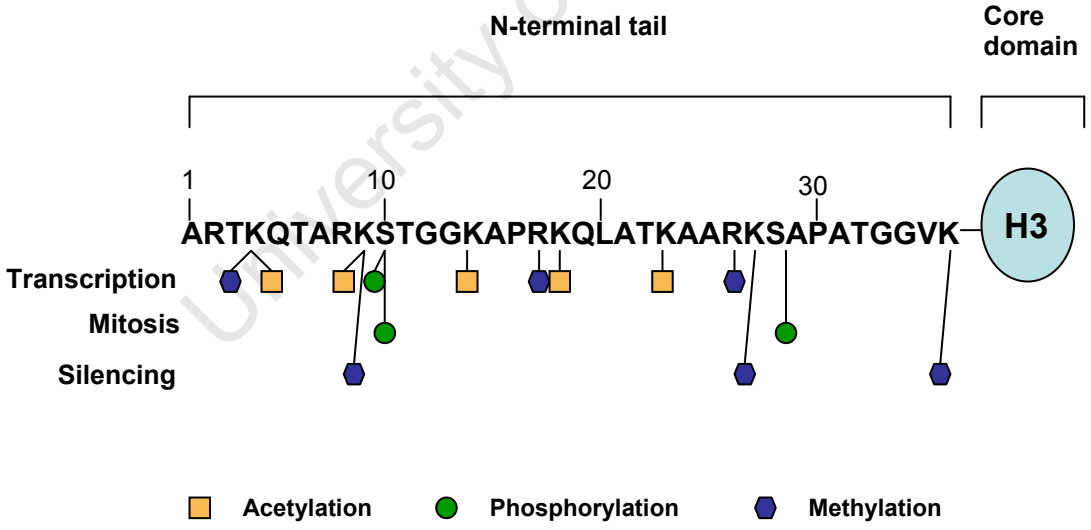



**Figure 1.5.** Post-translational modification of the histone H3 N-terminal tail domain. The potential sites of the covalent modifications, acetylation, phosphorylation and methylation, are indicated and the cellular processes affected are shown on the left (adapted from Nowak and Corces, 2004).

### 1.5.2. Histone H3 phosphorylation during mitosis

Histone H3 phosphorylation at Ser10 was found to be associated with chromosome condensation and segregation during mitosis and has hence been regarded as a marker of mitosis. The pattern of mitotic histone H3-Ser10 phosphyorylation was shown to be global (Gurley et al., 1978; Hendzel et al., 1997; Johansen, 2006); begining in pericentric heterochromatin during G2 phase, becoming widespread by prophase, and persisting to telophase (Gurley et al., 1978; Hendzel et al., 1997). In addition, Ser28 phosphorylation of histone H3 was also identified as another mitotic marker. Members of the Aurora kinase family have been suggested to be responsible for this phosphorylation of Ser10 and Ser28 in histone H3 during mitosis (Johansen, 2006).

### 1.5.3. Histone H3 phosphorylation during transcriptional activation

In 1991, pioneering work by Mahadevan and colleagues revealed a close correlation between phosphorylation of histone H3-Ser10 and induction of IE genes such as c-jun and c-fos in response to growth factors and phorbol esters in mouse fibroblast cells. These findings led the authors to postulate that a link exists between the phosphorylation of histone H3-Ser10 and transcriptional activation (Mahadevan et al., 1991). Thus, phosphorylation of histone H3 is associated not only with mitosis but also with the activation of gene expression. This raises the question of its role in these processes which involve such different chromatin states. To date, it is well known that stimulus-mediated phosphorylation of histone H3 is transient and that it affects only a small fraction of total histone H3 protein. This is different to the situation observed during mitosis where the majority of histone H3 is phosphorylated (Clayton and Mahadevan, 2003; Johansen, 2006). Several kinases have been suggested to be responsible for phosphorylating histone H3 at Ser10 and Ser28 in association with gene expression and will be discussed under section 1.5.4.

Further evidence supporting the role of histone H3-Ser10 phosphorylation in the induction of gene expression has been obtained from several studies as detailed in the following examples. Firstly, phosphorylation of histone H3-Ser10 has been found to occur at transcriptionally active heat shock loci in Drosophila larval salivary glands (Nowak and Corces, 2000). Secondly, treatment of mouse ovarian granulosa cells by follicle-stimulating hormone yields rapid histone H3 phosphorylation which suggests a

potential role in the transcription of genes required for the establishment of a cellular differentiation program (DeManno et al., 1999). Stimuli-induced phosphorylation of histone H3 at Ser10 was also found in certain neuronal cells and correlated with IE gene expression (Crosio et al., 2000; Brami-Cherrier et al., 2007). In addition to early genes, transcriptional activation of several "late" inducible genes was also shown to be associated with phosphorylation of histone H3. These include IL-2-mediated *HDAC1* expression in mouse B6.1 cells (Hauser et al., 2002) and expression of telomerase reverse transcriptase in response to mitogens and stress in human T cells and fibroblasts (Ge et al., 2006). Thus, phosphorylation of histone H3-Ser10 has been implicated in the expression of a number of stimulus-induced genes and an increasing number of genes regulated in this way continue to be identified.

In addition to Ser10, stimulation by mitogens and stress also cause phosphorylation of histone H3 at Ser28. One study using antibodies specific to phosphorylated histone H3-Ser10 and H3-Ser28 demonstrated that phosphorylation of Ser10 and Ser28 occur separately on different H3 tails and are even associated with different chromatin fragments. Unlike the case for H3-Ser10, there is very little association between phosphorylation of H3-Ser28 and stimuli-induced IE genes in mouse fibroblasts (Dyson et al., 2003). Whether there are target genes regulated by phosphorylation of histone H3-Ser28 remains to be elucidated.

There is conflicting information in the literature regarding whether there is a synergistic role between acetylation and phosphorylation of histone H3. A number of reports have shown that both phosphorylation and acetylation occur on induced genes (Clayton and Mahadevan, 2003). Moreover, acetylation and phosphorylation have been shown to coexist in the same histone H3 tail (Cheung et al., 2000; Clayton et al., 2000). Support for a synergistic role between phosphorylation and acetylation of histone H3 comes from a study which showed that mouse fibroblasts stimulated with EGF resulted in both phosphorylation and acetylation of the c-fos gene (Cheung et al., 2000). There is also evidence that phosphorylation/acetylation (Ser10/Lys14) of histone H3 is necessary for the recruitment of general transcription factors (Agalioti et al., 2002).

Several lines of evidence have also suggested that phosphorylation and acetylation are two parallel events and reciprocally independent but that there may be a functional

synergy between the two protein modifications (Clayton and Mahadevan, 2003). This possibility is supported by observations that only histone H3 phosphorylation occurs at transcriptionally active heat shock loci (Nowak and Corces, 2000) and that glutamate induces histone H3 phosphorylation but not acetylation in striatal neurons (Brami-Cherrier et al., 2007). It seems likely, that while on the one hand, both acetylation and phosphorylation are required for sufficient transcriptional activation of some genes, on the other hand, transcriptional activation of other genes are associated with phosphorylation of H3 but independent of acetylation.

### 1.5.4. Cell signalling pathways and kinases regulating histone H3 phosphorylation

Several cell signalling pathways have been implicated in the phosphorylation of histone H3 in response to a variety of stimuli but the mitogen-activated protein (MAP) kinase signalling pathways are the best understood.

The MAP kinases represent one of the most important and well characterised families of kinases, which catalyse the phosphorylation of a wide range of substrates in response to diverse stimuli (Robinson and Cobb, 1997). The MAP kinase family is divided into four distinct subgroups: the extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK5/big MAP kinase 1 (BMK1) and the p38 group of protein kinases. ERK family members are mainly activated by mitogenic stimuli, while the JNK and p38 MAP kinases are activated mainly in response to stress stimuli or inflammatory cytokines (Roux and Blenis, 2004; Kyriakis and Avruch, 2001). The recently identified subgroup, ERK5, is activated by a combination of mitogenic stimuli and osmotic stress (Wang and Tournier, 2006). MAP kinases are activated via a highly conserved phospho-relay system composed of three sequentially activated protein kinases. The first kinase in this cascade is the MAP kinase kinase kinase (MAPKKK) which, in response to a specific stimulus, phosphorylates and activates the dual-specificity MAP kinase kinase (MAPKK) at specific serine/threonine residues. MAPKK in turn phosphorylates MAP kinase at threonine and tyrosine residues within the Thr-Xaa-Tyr motif, which is essential for complete activation of the MAP kinase. Once activated, the proline-directed serine/threonine MAP kinase phosphorylates a range of

target proteins, many of which are transcription factors, cytoplasmic substrates and other kinases (Robinson and Cobb, 1997; Garrington and Johnson, 1999).

Mitogen- and stress- induced phosphorylation of histone H3 has been demonstrated to be mediated by the Erk and/or p38 MAPK cascades in many different systems. There is however no evidence showing that phosphorylation of histone H3 is mediated by the JNK signalling pathway in response to stimuli (Clayton and Mahadevan, 2003). However, a recent study showed that SP600125, a small molecule inhibitor of JNK, reduced global histone H3-Ser10 phosphorylation in human hepatocellular HepG2 cells. Interestingly, the authors found SP600125-dependent hypophosphorylation of histone H3-Ser10 to be independent of MAPKs (Huang et al., 2006). This would suggest that either the SP600125 JNK inhibitor is not specific to JNK or that there are circumstances under which JNK can phosphorylate histone H3.

There are several kinases that have been proposed to phosphorylate histone H3 in response to mitogens or stress. These include mitogen- and stress-activated kinase 1/2 (MSK1/2) and ribosomal S6 kinase 2 (RSK2). Current evidence suggests that MSK1 and 2 are primary histone 3 kinases (Clayton and Mahadevan, 2003). Both MSK1 and 2 are located in the nucleus and are activated by either ERK1/2 or p38. MSK1 has been shown to activate histone H3 in vitro, and an inhibitor of MSK blocks the nucleosomal response in vivo (Thomson et al., 1999). Furthermore, mitogens and stress have a severely reduced or no effect on histone H3 phosphorylation in MSK1/2 knockout mice (Soloaga et al., 2003).

Protein Kinase C (PKC) is a major intracellular target of TPA and it is well established that in response to TPA, PKCs can activate the Ras/Raf/MEK/ERK MAPK pathway. PKC represents an important multigene family of serine/threonine kinases that can be activated by various extracellular and intracellular signals and have been involved in broad and diverse functions in many physiological and pathological processes (Mellor and Parker, 1998; Newton et al., 1997; Griner and Kazanietz, 2007 ). The PKC family comprises at least 12 isoforms that are divided into three subfamilies: conventional or classical PKCs (cPKCs; α, β 1/2 and γ), novel PKCs (nPKCs; δ, ε, η, and θ), and atypical PKCs (aPKCs; ζ and λ/ι). There is also accumulating evidence suggesting that

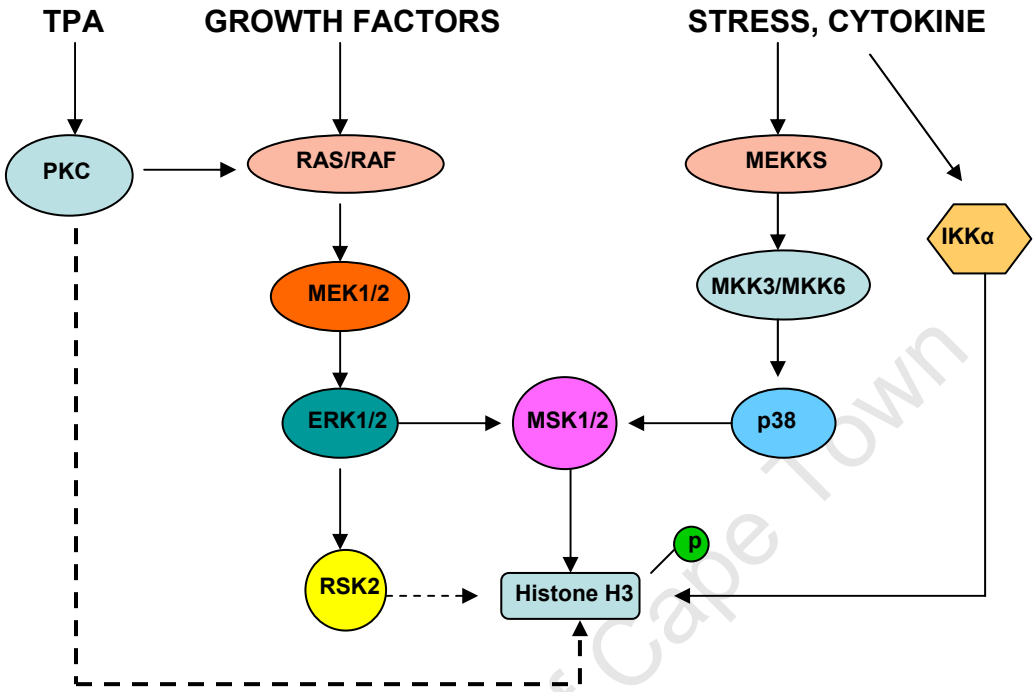



**Figure 1.6.** Schematic representation of the cell signalling pathways leading to histone H3 phosphorylation. Two well-established MAKP signalling pathways, ERK and p38, mediate histone H3 phosphorylatiion by activation of MSK1/2 in response to mitogens, growth factors, stress and cytokines. The IKK-α is responsible for phosphorylating histone H3 in cytokine-induced activation of NF-KB transcription. PKC may also act as a histone H3 kinase.

TPA can activate p38 and JNK in a cell-type dependent manner (López-Bergami et al., 2005; Mauro et al., 2002). Moreover, phorbol esters have also been shown to induce histone H3-Ser10 phosphorylation at the LDL receptor promoter in a protein kinase C-dependent manner (Huang et al., 2004).

It is worth noting that cytokines induce the expression of several NF-kB-dependent genes via activation of the NF-kB pathway. IkB kinase α (IKK-α), a downstream component of this pathway, has been suggested to play a role in phosphorylating histone H3 (Yamamoto et al., 2003; Anest et al., 2003). Protein kinase A (PKA) also mediates phosphorylation of histone H3-Ser10 in rat ovarian granulose cells treated with FSH (DeManno et al., 1999) (Fig. 1.6.).

As described above, phosphorylation of histone H3 has been associated with the regulation of gene expresson, and several signalling pathways have been identified that mediate H3 phosphorylation. However, stimulus-induced histone H3 phosphorylation is not sufficient for the induction of expression of some genes and thus, phosphorylation of H3 may cooperate with other molecular mechanisms that regulate genes such as transcription factor phosphorylation.



## 1.6 General and specific aims of study

Tbx2 has been implicated in several developmental processes such as coordinating cell fate, patterning and morphogenesis of a wide range of tissues and organs. Furthermore, Tbx2 is also emerging as a key regulator of the cell cycle and dosage sensitivity of this gene has been implicated in several cancers. However very little is also known about the signalling pathways regulating the expression and activity of Tbx2. While its role as a transcriptional repressor is well defined there is limited information regarding its target genes. Identifying Tbx2 target genes as well as signalling pathways that regulate the activity of Tbx2 is imperative to understanding its oncogenic role and may provide important targets for cancer treatments.The general **aim** of this project is therefore to explore the ability of Tbx2 to regulate the type 1 collagen gene and to investigate signalling pathways that regulate Tbx2 expression. This will be achieved by the following broad aims:

1. To investigate the regulation of type 1 collagen gene expression by Tbx2.
2. To clone the human Tbx2 regulatory region and to identify cis-acting elements involved in the basal transcription of the Tbx2 gene.
3. To investigate the regulation of Tbx2 gene expression by signalling pathways.

# CHAPTER 2

# A ROLE FOR TBX2 IN THE REGULATION OF THE α2(1) COLLAGEN GENE IN HUMAN FIBROBLASTS

## 2.1. Introduction

Recent studies have implicated the mouse type 1 collagen gene as a potential Tbx2 target (Chen et al., 2001). Type I collagen synthesis is crucial for normal embryonic development and in maintaining tissue integrity and its aberrant expression has deleterious effects on several biological processes (Bornstein and Sage, 1989). Mice lacking the α1(I) collagen gene for example, display defective angiogenesis (Lohhler et al., 1984), while in humans, dominant negative mutations in any of the type I collagen genes result in diseases such as osteogenesis imperfecta and Ehlers Danlos syndrome (Kuivaniemi et al., 1991; Prockop et al., 1993, 1994). Abnormal collagen synthesis is also associated with diseases such as arthritis, fibrosis and with tumour cell invasion and metastasis (Fenhalls et al., 1999; Fusenig et al., 2004). In an attempt to identify genes that may be regulated by Tbx2, DNA microarray analysis was performed on mouse NIH3T3 fibroblasts overexpressing *Tbx2* and the results revealed that the type 1 collagen gene was upregulated (Chen et al., 2001). Interestingly, a parallel investigation in which *Tbx2* was overexpressed in the rat ROS17/2.8 osteoblastic cell line showed down-regulation of type I collagen (Chen et al., 2001). While this study suggests that Tbx2 may function as both activator and repressor, the data does not address whether Tbx2 directly mediates the transcriptional effect on the type 1 collagen gene. However, these contrasting results do suggest that the cell context and/or the species may be important in determining the effect of Tbx2 on the expression of the type I collagen genes.

The aim of this chapter was therefore to investigate the regulation of type 1 collagen gene expression by Tbx2. Our findings show that there is a correlation between the expression patterns of endogenous Tbx2 and *COL1A2* in several human fibroblast cell lines and provide compelling evidence that Tbx2 represses expression of the human *COL1A2* gene. This study suggests that Tbx2 is involved in the regulation of the human *COL1A2* gene which has important implications for our understanding of the role of Tbx2 in development and cancer.

## 2.2. Results

### 2.2.1. Correlation between TBX2 and COL1A2 expression patterns in fibroblast cell lines

To determine whether Tbx2 can regulate collagen gene expression we firstly investigated whether TBX2 was expressed in normal and transformed fibroblast cell lines. The results of western blot analyses (Fig. 2.1A) show that TBX2 was expressed at high levels in the normal WI-38 fibroblast cell line, was dramatically reduced in WI-38 cells transformed by exposure to cobalt radiation (CT-1) and was almost undetectable in the SV40-transformed WI-38 cells (SVWI-38) and in human fibrosarcoma cells (HT1080). Interestingly, this pattern of TBX2 expression followed the same trend as that obtained for COL1A2 mRNA (Fig. 2.1B). This direct correlation initially suggested that Tbx2 may play a role in activating the human *COL1A2* promoter.

### 2.2.2. Overexpression of TBX2 reduces COL1A2 mRNA levels

To determine whether Tbx2 regulates endogenous *COL1A2* gene expression, we established CT-1 and HT1080 cell lines that stably express exogenous Tbx2. The CT-1 cell line was selected because it had reduced Tbx2 protein levels and reduced COL1A2 mRNA compared to the normal parental WI-38 cells. The HT1080 cell line, on the other hand, was chosen to determine whether the introduction of Tbx2 would be sufficient to induce *COL1A2* gene expression because it lacked detectable levels of COL1A2 mRNA. A number of G418-resistant clones were tested for TBX2 expression. Figure 2.2.A shows the presence of Tbx2 protein in representative clones (CT-Tbx2(2), CT-Tbx2(3), HT-Tbx2(5) and HT-Tbx2(6)) that is undetectable in cells transfected with the empty vector (CT-E and HT-E). This result was confirmed by immunocytochemistry (Fig. 2.2.B) where predominant staining was seen in the nuclei of CT-Tbx2 and HT-Tbx2 cell lines while low levels were detected in CT-E cells and no immunostaining was observed in the control HT1080 cell line harboring empty vector pcDNA (data not shown). We selected the CT-Tbx2(2), CT-Tbx2(3), HT-Tbx2(5) and HT-Tbx2(6) cell lines to investigate the effect of Tbx2 on endogenous *COL1A2* using qRT-PCR. Our results, shown in Figure 2.2.C, indicate that the COL1A2 mRNA levels were repressed by approximately 2 fold in the two CT-Tbx2 cell lines compared to the control CT-E cells. This suggests that

overexpression of Tbx2 leads to downregulation of *COL1A2* gene expression. These results confirmed that the *COL1A2* gene is repressed independently of Tbx2 in the HT1080 cells. Taken together these results raised the question of whether Tbx2 was able to directly regulate expression of the *COL1A2* gene.

### 2.2.3. Tbx2 represses COL1A2 promoter activity

Tbx2 has been shown to regulate target gene expression by binding the consensus T-box binding site GTGTGA as well as the GTGTTA and GGGTGA sequences. We therefore screened the 5' upstream regulatory region of the human α2(I) collagen gene for these sites and located a GTGTGA sequence at position -1400. To determine whether Tbx2 downregulates the *COL1A2* gene directly through this site, we performed transient transfection assays using a luciferase reporter driven by 5' deletion constructs of the human *COL1A2* gene promoter (Fig. 2.3.A). HT1080 cells were initially used in these assays because they do not have detectable levels of endogenous Tbx2 protein or collagen mRNA. The transfection data showed that wild-type Tbx2 acts as a strong dose-dependent repressor of the *COL1A2* promoter (Fig. 2.3.B). All four human *COL1A2* promoter-deletion constructs tested appear to be strongly repressed by Tbx2 suggesting that Tbx2 acts through an element located in the region -107 to +50 of the human *COL1A2* promoter and not through the putative binding site at position -1400. Similar results were obtained in CT-1 cells that have detectable levels of COL1A2 mRNA and Tbx2 protein (Fig. 2.3.C). Further investigation of the Tbx2 response element by deletion mapping was prevented due to loss of basal promoter activity.

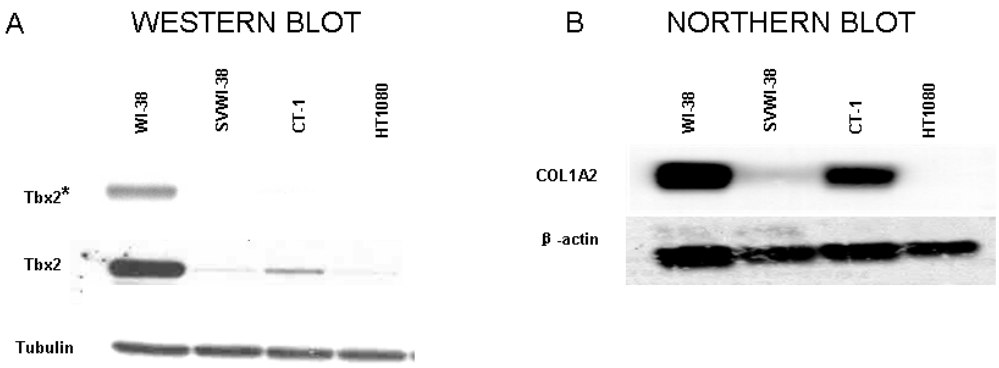
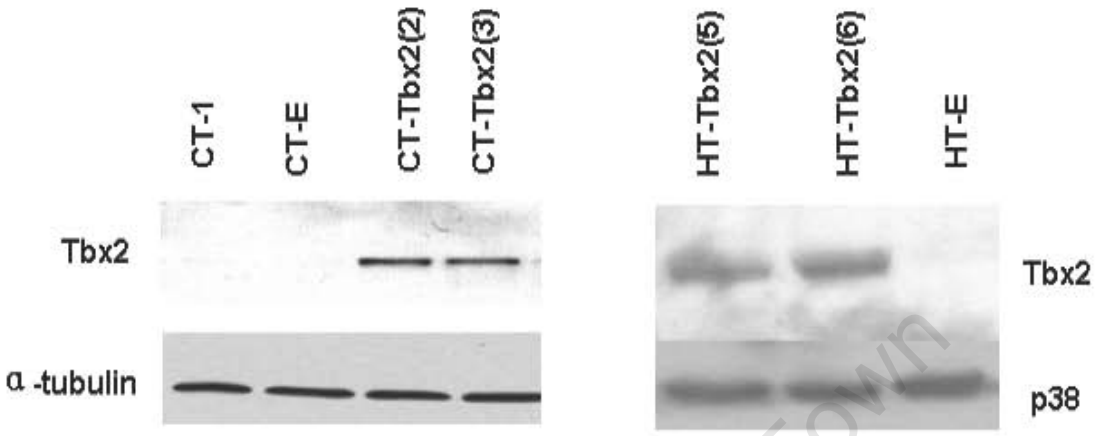



**Figure 2.1**. Tbx2 protein and COL1A2 mRNA exhibit similar patterns of expression in normal and transformed human fibroblast cell lines. (A) Western blot analyses were used to compare the relative levels of Tbx2 protein in normal embryonic lung fibroblasts (WI-38 cell line), two of its transformed counterparts (SVWI-38 and CT-1) and a naturally occurring fibrosarcoma cell line (HT1080). Protein blots were probed with antibodies specific for Tbx2 and tubulin and detected by enhanced chemiluminescence as described in Materials and methods. * indicate a short exposure of the blot. (B) Northern blot analyses, using total RNA (5µg) from cell lines described in (A) were used to compare the levels of COL1A2 mRNA. Blots were hybridised with probes to COL1A2 and β-actin.

**A**




**B**

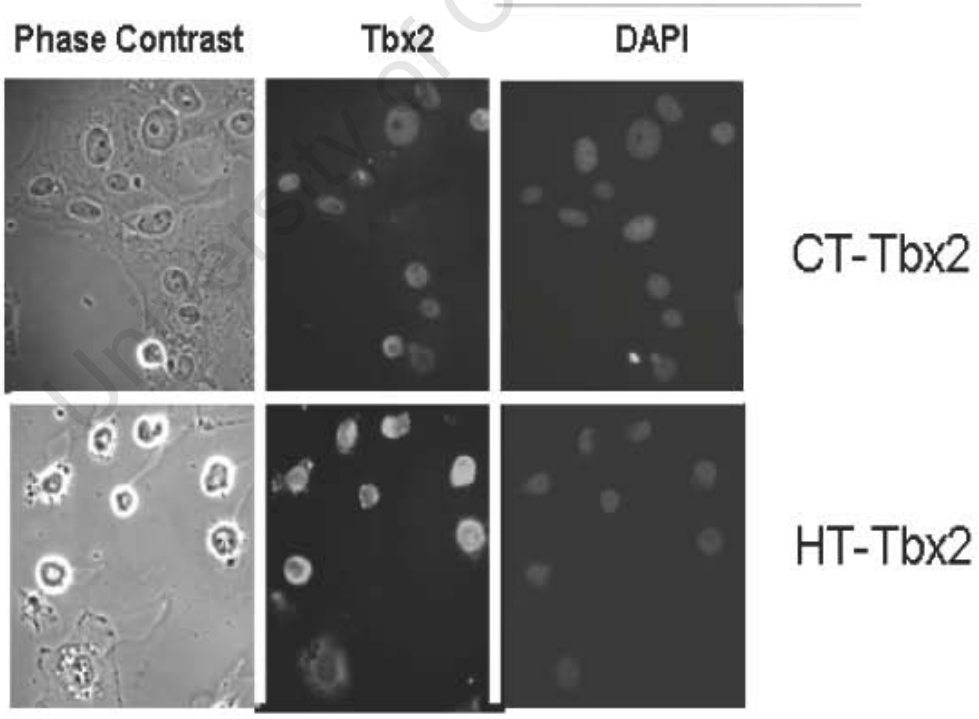





Figure 2.2. See overleaf for legend

**C**

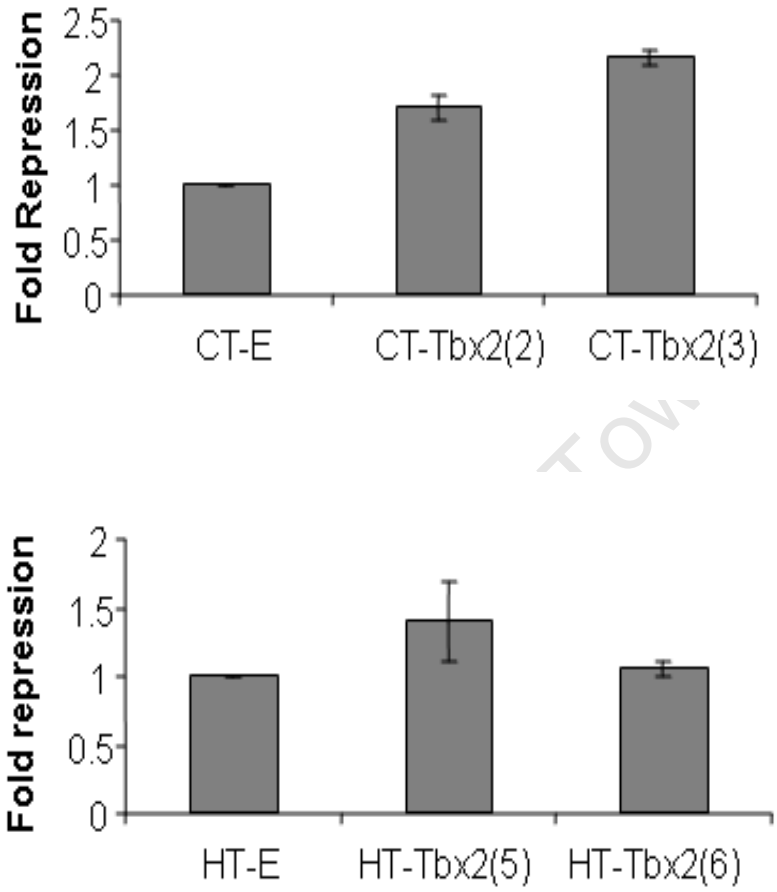



**Figure 2.2.** Tbx2 represses endogenous *COL1A2*. CT-E, CT-Tbx2, HT-E and HT-Tbx2 cell lines were established by stable transfection of CT-1 and HT1080 cells with either an empty vector or a TBX2-expression plasmid followed by G418 selection. (A) Western blot and (B) Immunohistochemical analyses were used to confirm that the CT-Tbx2 and HT-Tbx2 cell lines express readily detectable levels of Tbx2 protein. Both tubulin and total p38 levels were used as loading controls as similar studies have shown that both markers give comparable results. (C) Quantitative real-time PCR was performed to establish the effect of stably expressing TBX2 on *COL1A2* in CT-1 and HT-1080 cells. (C) Total RNA was extracted from CT-1, CT-E, CT-Tbx2(2), CT-Tbx2(3), HT-E, HT-Tbx2(5) and HT-Tbx2(6). Quantitative real-time PCR was then performed on reverse transcribed RNA using primers specific to COL1A2 and mRNA levels were normalised to GAPDH.

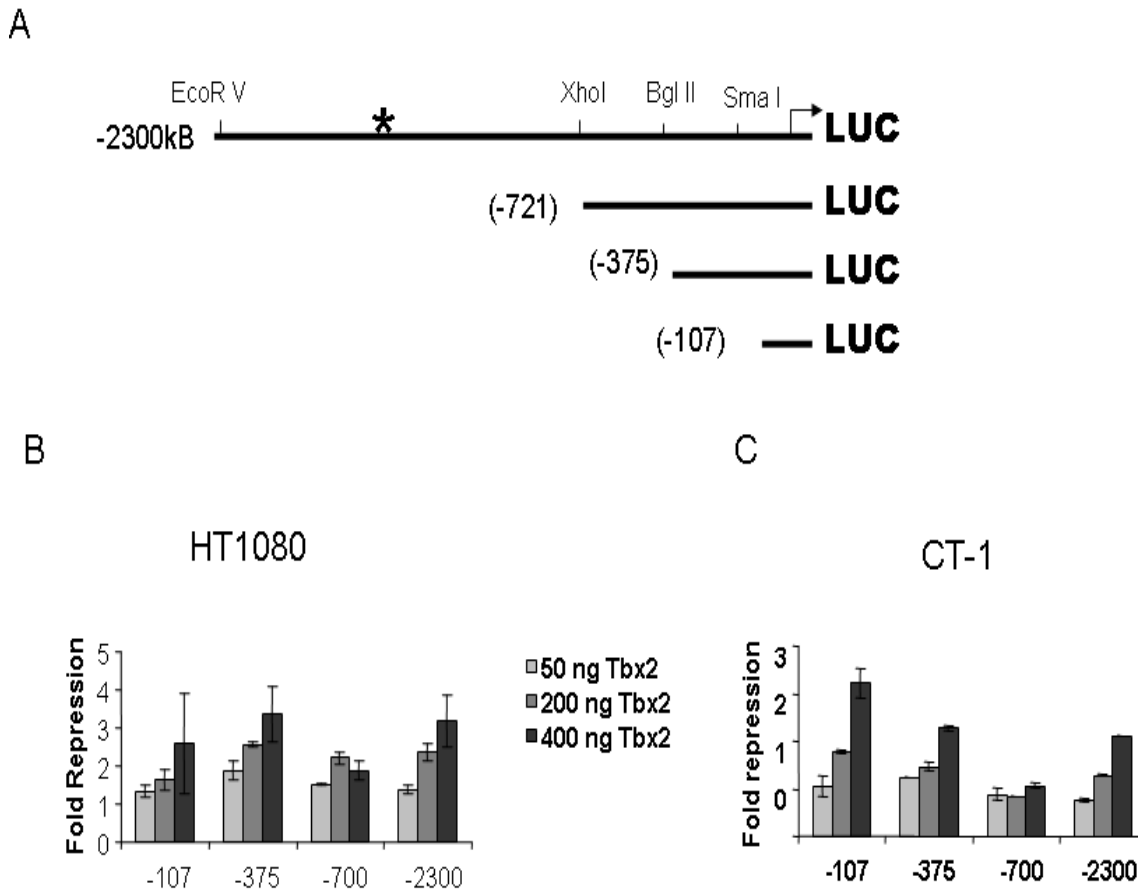





**Figure 2.3.** Tbx2 represses *COL1A2* promoter activity. (A) Schematic representation of *COL1A2* 5' deletion constructs generated by restriction enzyme digestion. The arrow indicates the transcription start site at +1 and the * indicates the putative Tbx2 binding site (GTGTGA) at -1400. The plasmids containing sequentially deleted fragments of p(-2300)Luc were transiently transfected into the HT1080 (B) and CT-1 (C) cell lines together with increasing amounts of the Tbx2 expression plasmid pCMV-Tbx2. Total amount of plasmid DNA transfected was held constant using the corresponding empty vector, pCMV. The plasmid pRL-TK containing the Renilla luciferase reporter gene was also introduced to normalize transfection efficiency. Promoter activity is indicated as fold repression which represents the ratio of the luciferase activity generated by the pCMV empty vector (without Tbx2) to that obtained in the presence of pCMV-Tbx2.

## 2.3. Discussion

Type I collagen is the most abundant protein in nature and plays a very important role during development as it is the basic component of skin, cartilage, bone and connective tissue (Kadler et al., 1996). Appropriate regulation of expression of the Type I collagen gene is therefore crucial. The transcription factor, Tbx2, is essential for normal embryonic development, most probably by regulating the expression of developmentally important genes such as those that encode the collagens. Although the expression patterns of Tbx2 during development have been widely studied, only a few of its target genes have been identified to date (Carreira et al., 1998; Chen et al., 2001; Jacobs et al., 2000; Prince et al., 2004). This study shows that TBX2 is expressed in human lung fibroblasts and is downregulated in transformed fibroblasts in a manner which mimics the expression pattern for the *COL1A2* gene. This initial observation suggested that Tbx2 may be acting as a positive regulator of the *COL1A2* gene. However, further investigations showed that Tbx2 is in fact a negative regulator of the *COL1A2* gene and that it is able to repress its expression in the absence of the published consensus T-element. This result is consistent with that obtained by Jacobs et al. (2000) who also found that Tbx2 was able to repress the $p19^{ARF}$ promoter in the absence of a putative T-box binding site. Taken together, these results suggest that Tbx2 may be functioning as a co-repressor of the *COL1A2* gene which is in keeping with the suggestion that the target specificity of T-box family members depends on their association with different cofactors. Thus, an important research approach would be to investigate proteins which may interact with Tbx2 by, for example, the yeast two-hybrid screen assay. It is of course also possible that the effect seen on *COL1A2* is due to the repression by Tbx2 of a *COL1A2* activator.

The involvement of Tbx2 in tumourigenesis is poorly defined and it is not clear whether it is involved in the initiation, progression and/or metastasis of cancer. Tumour invasion and metastasis require breakdown of collagen and a reduction in collagen production (Christner et al., 2006; Liotta et al., 1979; Tanjore and Kalluri, 2006). It would be important to clarify whether the ability of Tbx2 to repress *COL1A2* has any significance in these processes. We cannot, however, rule out the possibility that the repression of the endogenous *COL1A2* gene in the CT-Tbx2 cell lines may have resulted from other cellular changes induced by Tbx2. The effect of Tbx2 on the *COL1A2* promoter in

luciferase assays would however suggest that at the very least Tbx2 is able to directly regulate the expression of this gene as a co-repressor. Identifying candidate Tbx2 accessory proteins involved in regulating *COL1A2* gene expression may provide important clues as to the precise mechanism by which Tbx2 regulates this important gene.

Having identified *COL1A2* as a Tbx2 target gene, we next investigated the regulation of the TBX2 gene by identifying cis-acting elements important for TBX2 expression as well as common signalling pathways regulating both genes.

## 2.4. Materials and methods

### 2.4.1. Cell Culture

Human embryonic lung fibroblast WI-38 cells (ATCC CCL-75), SV40 transformed WI-38 fibroblast cells (SVWI-38) (de Haan et al., 1986), γ-radiation transformed WI-38 fibroblast cells (CT-1) (Namba et al., 1980) and human fibrosarcoma cells HT1080 (HT) (ATCC CCL-120) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100μg/ml streptomycin at 37ºC in an atmosphere of 5% $CO_2$.

### 2.4.2. Generation of Stable Cell Lines Expressing Tbx2

To generate stably transfected cell lines, CT-1 and HT1080 cells were seeded in duplicate in 60-mm dishes at a density of 3.2 X $10^5$ cells per dish 24 h before transfection. Cells were transfected with either the empty expression vector pcDNA3.1 (+) or with this vector containing the full length human Tbx2 cDNA (Lingbeek et al., 2002) using the standard calcium-phosphate precipitation method. Transfected cells were allowed to recover for 48 h and CT-1 and HT1080 transfectants were selected in medium containing 400 μg/ml and 800 μg/ml G418 respectively. Colonies were subcloned and selected based on positive Tbx2 immunoreactivity in the case of pcDNA3.1-Tbx2 transfectants. Among subcloned cell lines, two from both the CT-1 and HT1080 cell lines were chosen for subsequent analysis. CT-Tbx2 and HT-Tbx2 contained the pcDNA3.1-Tbx2 construct while CT-E and HT-E contained pcDNA3.1 empty vector.

### 2.4.3. Plasmid Constructs

The human *COL1A2* promoter luciferase reporters were generated by inserting the EcoRV(-2389)-Hind III(+58), Xho 1(-721)-Hind III (+58), Bgl II (-375)-Hind III (+58) and Sma I (-107)-Hind III (+58) fragments of the *COL1A2* gene (Parker et al., 1992) into the appropriately cleaved luciferase reporter vector pRL-CMV-basic (Promega, USA). The pRL-CMV vector (Promega, USA) was used as an internal control reporter to test for

transfection efficiency. The pcDNA3.1-Tbx2 expression vector was kindly provided by Dr. Merel Lingbeek (Lingbeek et al., 2002)

### 2.4.4. Transient Transfection Assay

Cells were plated at 1.5 x $10^5$ cells/ml in six-well plates 1 day before transfection. Non-liposomal mediated gene transfer was performed using FuGENE®6 (Roche Applied Science) according to manufacturer's instructions using 1 μg of DNA consisting of the reporter construct, the Tbx2 expression vector and the internal control vector. Thirty hours after transfection, cells were analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay (Promega, USA) following manufacturer's instructions and quantified with a Luminoskan Ascent Luminometer (Thermo LabSystems, USA).

### 2.4.5. Western blot analyses

Cells were harvested and solubilized at 4°C with lysis buffer (40 mM Tris-HCl, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1% Nonidet P40, 0.1% SDS and protease inhibitors), incubated on ice for 30 minutes and centrifuged at 12000 rpm for 20 minutes at 4°C. Protein concentrations in lysates were determined using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. Twenty micrograms of protein extract were separated on a 10% SDS-PAGE gel and then transferred onto nitrocellulose Hybond-C membrane (Amersham, USA). Following blocking for 1 hour at room temperature, the membranes were probed with mouse monoclonal anti-Tbx2 primary antibody (62-2; 1:2500). Immunoreactive bands were visualized with a horseradish peroxidase-conjugated secondary goat anti-mouse serum (1:4000) (Biorad, USA) and detected with enhanced chemiluminescence (ECL) (Pierce, USA). Monoclonal mouse anti-α-tubulin (1:500, Santa Cruz Biotechnology, USA) and rabbit polyclonal anti-p38 (1:5000, Cell Signalling Technology Inc., Beverly, MA) primary antibodies were used for normalization.

### 2.4.6. Northern blot analyses

Total RNA was extracted from cultured cells using Trizol reagent (Life Technologies, USA). RNA concentration was determined by spectrophotometric absorbance at 260 nm, and 5 μg was separated by electrophoresis in 1% formaldehyde-agarose gels. Gels were then transferred by capillary action to nylon membranes (Amersham, USA). Membranes were probed with random-primed $^{32}$P-radiolabeled (Amersham, USA) *Col1A2* and β-actin cDNA fragments. Hybridization was carried out in the ULTRAhyb buffer (Ambion, UK) following the manufacturer's instructions.

### 2.4.7. Microscopy

Cells grown on glass coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min and permeabilized in 0.2% Triton X-100 in phosphate buffered saline (PBS) for 10 min. Cells were incubated overnight with mouse Tbx2 monoclonal antibody (62-2) at a dilution of 1:750 and then incubated with the appropriate secondary antibody coupled to alexa 488 (Molecular Probes, USA) at a 1:1000 dilution. Cells were incubated in the dark with 1 μg/ml DAPI in PBS for 10 min, mounted on a slide and visualized by fluorescence microscopy.



### 2.4.8. Real time RT PCR

Total cellular RNA was extracted as described for northern blot analyses. Reverse transcription was carried out using Superscript III Reverse Transcriptase (Invitrogen), with oligo(dT)$_{20}$ primers. PCR was conducted using 2 μl of a 1 in 10 dilution of the cDNA and primers (*COL1A2*: forward 5'-GATTGAGACCCTTCTTACTCCTGAA-3'; reverse 5'-GGGTGGCTGAGTCTCAAGTCA-3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-GAAGGCTGGGGCTCATTT-3'; reverse 5'-CAGGAGGCATTGCTGATGAT-3') with LightCycler FastStart DNA Master$^{PLUS}$ SYBR green 1 kit (Roche) according to the manufacturer's protocol. Real-time PCR was carried out on a LightCycler Version 3 (Roche) with the following protocol: 95°C for 10 min; and 45 cycles of 95°C for 10 seconds (denaturation), 55°C for 5 seconds (annealing), and 72°C for 10 seconds (extension), followed by a standard melting curve program. Each

DNA sample was quantified in duplicate and a negative control without cDNA template was run with every assay to assess the overall specificity. Melting curve analyses was carried out to ensure product specificity and data was analysed using the $2^{-\Delta\Delta Ct}$ method. COL1A2 mRNA levels were normalized to GAPDH with PCR efficiency correction calculated using the formula Ratio = $(Etarget)^{CPtarget(control - sample)}/(Eref)^{CPref(control - sample)}$ ; E: real-time PCR efficiency, CP: crossing-point (Pfaffl, 2001).

# CHAPTER 3:

# CLONING OF THE 5' REGULATORY REGION OF THE HUMAN TBX2 GENE AND FUNCTIONAL CHARACTERIZATION OF CIS-ACTING ELEMENTS INVOLVED IN ITS BASAL TRANSCRIPTION

## 3.1. Introduction

Elucidating the mechanisms regulating Tbx2 expression is of importance for understanding the precise roles of Tbx2 in physiological and pathological processes. It is thus surprising that to date relatively little is known about the transcriptional regulation of the Tbx2 gene.

The 5'-flanking region of the mouse *Tbx2* gene has been cloned and the transcription factor Mitf was shown to activate *Tbx2* gene expression by recognizing an E-box element in melanocytes (Carreira et al., 2000). A putative retinoic acid response element was also identified in this promoter and it was shown to be responsible for retinoic acid stimulated *Tbx2* gene expression (Boskovic and Niles, 2004). A recent study showed that Tbx20 was able to bind in vivo to a 5'-flanking regulatory region of the mouse *Tbx2* gene which contains a putative T-element. This finding suggested that *Tbx2* is a direct target for repression by Tbx20 in the developing heart (Cai et al., 2005). However, important cis-acting elements responsible for Tbx2 gene expression have not yet been identified, and relatively few studies have reported on the regulation of the human TBX2 gene.

To better understand the molecular mechanisms regulating Tbx2 gene expression, one of the aims of this study was to clone and characterize the 5'-regulatory region of the TBX2 gene and the details are described in this chapter. Having successfully cloned a 5'-flanking region of the TBX2 gene from the human WI-38 fibroblasts, we show that the proximal promoter (-216/+32) mediates most of the basal activity and that a proximal Sp1 site juxtaposed to an inverted CCAAT box element are both essential for basal promoter activity. While a physical and functional interaction between Sp1 and the CCAAT box binding protein NF-Y was not essential for optimal basal promoter activity, a dynamic balance in their levels appears to be important. Furthermore, our data reveal a potential downstream promoter element (DPE) in the TBX2 promoter which significantly influences basal activity.

## 3.2. Results

### 3.2.1. Cloning and functional analysis of the 5'- regulatory region of the TBX2 gene

To identify potential cis-acting elements required for basal TBX2 gene activity, a 1638 bp DNA fragment from -1606 to +32, relative to a putative transcriptional start site was cloned (Fig. 3.1.A). The whole sequence was verified by sequencing (see appendix 1) and confirmed by alignment with the human genomic DNA database. A 98% nucleotide identity was observed between the -216 to +32 region of the human TBX2 gene and the mouse *Tbx2* proximal promoter (Fig. 3.1.B).

To define the region essential for basal transcription of the TBX2 gene, a series of 5'-deletion luciferase reporter constructs were prepared using restriction enzymes as shown in Fig. 3.1.A. Functional analysis of these constructs showed that a proximal promoter (-216/+32) maintains high levels of basal activity (Fig. 3.1.C). This together with the observation that this region shares a very high degree of homology with the mouse *Tbx2* proximal promoter suggested that it will contained important cis-acting elements. We, therefore, used the TFSEARCH program (www.cbrc.jp/research/db/TFSEARCH.html) to search for potential cis-acting elements in this proximal promoter and putative regulatory elements are shown in Fig. 3.1.B. To determine whether these putative elements were responsible for basal activity of the TBX2 gene, mutant constructs for each of the putative sites were generated and luciferase activity assays were carried out. Whereas individual mutations in the Sp1 and CCAAT box sites resulted in a 6.7-fold and a 4.6-fold decrease of luciferase activity respectively, mutating both sites resulted in a 13.5-fold reduction (Fig. 3.1.D). Mutating the other putative sites individually did not significantly affect basal promoter activity (see appendix 4).

### 3.2.2. Sp1 and NF-Y bind the Sp1 and CCAAT box motifs respectively identified in the TBX2 proximal promoter

The ubiquitously expressed Sp1 and NF-Y transcription factors are potentially able to regulate the proximal TBX2 promoter through binding the identified Sp1 and CCAAT box sites respectively. To address this possibility, we carried out gel shift assays and the

results indicated that nuclear factors bound to radiolabelled oligonucleotides which contain either only the wild type CCAAT box motif (Fig.3.2.A) or Sp1 motif (Fig. 3.2.B). A 50-fold molar excess of unlabelled oligonucleotides efficiently competed for binding of nuclear factors. Furthermore, supershift assays confirmed that Sp1 and NF-Y did bind specifically to the Sp1 and CCAAT box motifs respectively. ChIP assays demonstrate that Sp1 and NF-Y bind the TBX2 proximal promoter in WI-38 human lung fibroblasts suggesting that they may be required for constitutive expression of TBX2 in these cells (Fig. 3.2.D).

### 3.2.3. Sp1 binds the proximal TBX2 promoter regardless of the status of the CCAAT box

To investigate whether a physical interaction between Sp1 and NF-Y influences the binding of Sp1 to its cognate site, a DNA affinity immunoblot assay (DAI) was performed. Considering that the context of DNA may affect binding, two biotinylated probes of 170 bp which contained the wild-type Sp1 and either the wild-type or mutant CCAAT box motif were generated by PCR and used in this assay. The results showed that a similar amount of Sp1 bound to both probes suggesting that the integrity of the CCAAT box motif did not affect its binding (Fig. 3.2.E). This is further illustrated by the observation that in the same experiment only unlabeled probes containing WT Sp1 motifs, regardless of the CCAAT box status, competed well for binding. To further explore the mechanism by which Sp1 transcriptionally regulates the TBX2 gene, co-transfection experiments were carried out with a Sp1 expression vector and either a wild type- or CCAAT box mutated TBX2 proximal promoter (-216/+32) luciferase construct. It is interesting to note that in these experiments the over-expression of Sp1 did not significantly activate the wild-type promoter, but led to a 2.6-fold activation of the CCAAT box mutant promoter (Fig. 3.2.F).

### 3.2.4. A potential role for a DPE motif in the proximal promoter of the TBX2 gene

To identify potential core promoter elements the proximal TBX2 promoter was screened for putative transcription factor binding sites (shown in Fig. 3.3.A) which were individually mutated. Luciferase assays show that none of these sites affected basal TBX2 promoter

activity (Fig. 3.3.B). Interestingly a downstream core promoter element (DPE)-like motif (AGTCGCG) was found to be located from +26 to +32 and a 12bp deletion construct (from +20 to +32) exhibited a ~25% reduction in luciferase activity compared to the WT-TBX2 proximal promoter. This DPE-like motif may thus also play a role in basal TBX2 promoter activity.

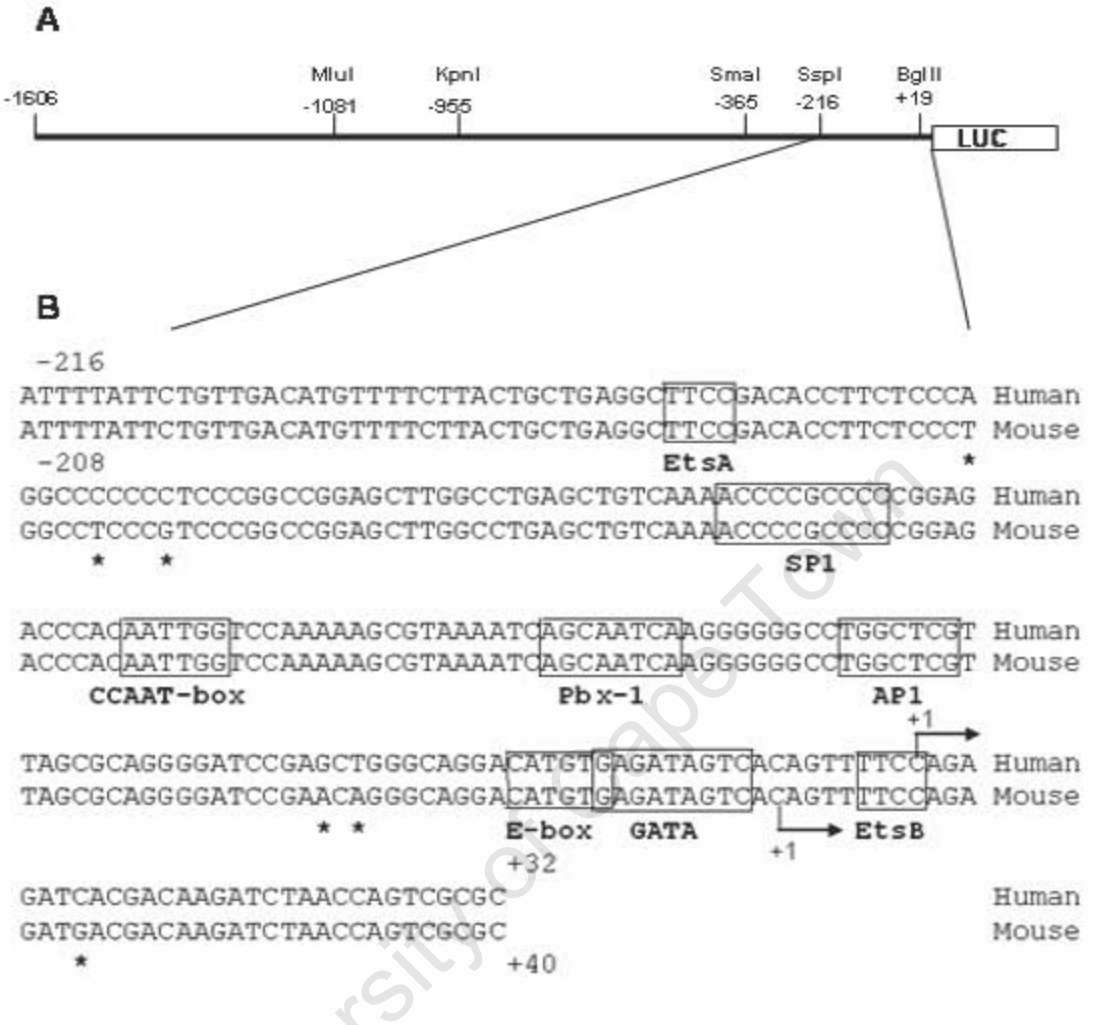





Figure 3.1 See overleaf for legend

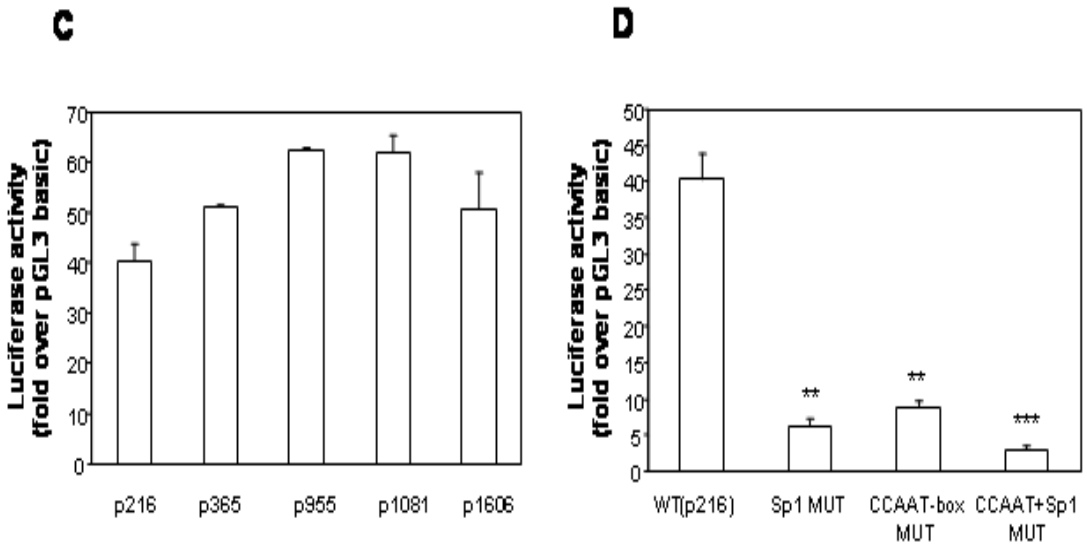





**Fig. 3.1.** The proximal promoter of the human TBX2 gene (-216/+32) is sufficient for maintaining the majority of basal activity. (A) Schematic illustration showing the restriction enzymes used to generate a series of luciferase reporter constructs containing truncated 5'-flanking regulatory regions of the human TBX2 gene. (B) Sequence alignment showing homology between the human (top) and mouse (low) Tbx2 proximal gene promoter. The asterisks denote nucleotides that deviate between human and mouse. Putative cis-regulatory elements are boxed. Nucleotides are numbered relative to the transcription initiation site (+1). Sequence database are from http://www.ensemble.org. (C) Plasmids containing sequentially deleted fragments of the human TBX2 promoter-luciferase reporter (500 ng) were transiently transfected into the HT1080 cell line together with the plasmid pRL-TK (50 ng) containing the Renilla luciferase reporter gene. (D) 500 ng proximal promoter construct (-216/+32) and its mutants (Sp1MUT, CCAAT-box MUT, and Sp1+CCAAT MUT) were co-transfected with 50 ng internal control pRL-TK into HT1080 cells respectively. Firefly luciferase activity was normalized to renilla luciferase activity. Activation fold values were calculated by setting wild type promoter activity to 1. Values represented are mean(±) from three independent experiments. **, $p<0.01$, ***, $p< 0.005$

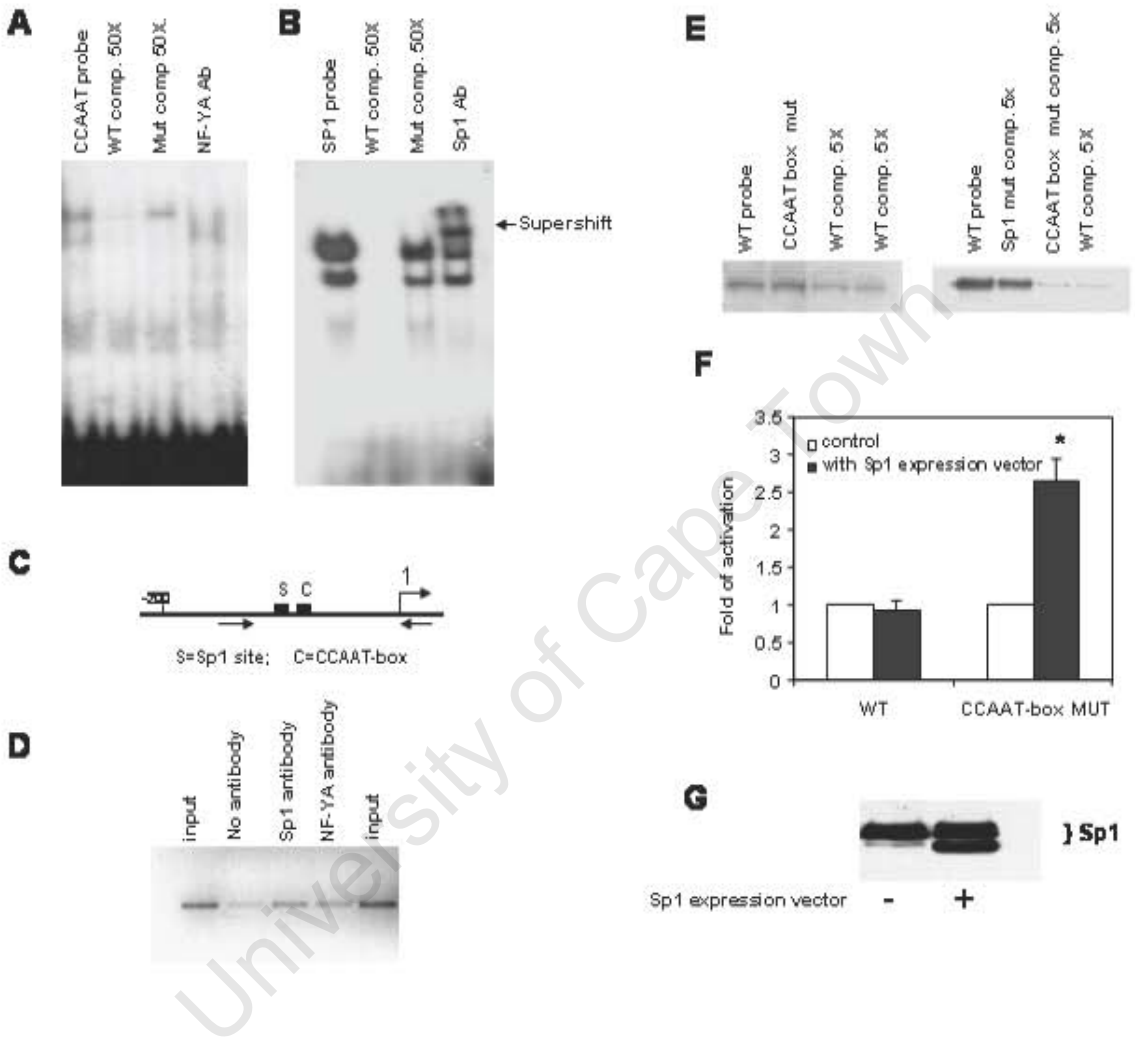





Figure 3.2 See overleaf for legend

**Fig. 3.2.** Sp1 and NF-Y bind the TBX2 promoter at Sp1 and CCAAT motifs respectively. (A, B) For EMSA, $^{32}$P-labeled double-stranded oligonucleotide probes containing either the Sp1 site or the CCAAT-box respectively were incubated with 5 µg of nuclear extracts from WI-38 cells. Competition analysis was carried out in the presence of a 50-fold molar excess of unlabeled wild-type or mutated probes. For supershift assay, antibodies for Sp1 or NF-YA were included in the reactions and the arrow indicates a supershift band. (C) Schematic illustration of the region of the TBX2 proximal promoter with the positions of the putative Sp1 and CCAAT-box motifs are represented as black boxes. (D) ChIP assays were performed with antibodies for Sp1 or NF-YA, and PCR was performed with the primer pairs indicated by arrows in (C) above. Inputs and no antibody are shown as positive and negative controls respectively. (E) For DAI analysis, biotinylated wild-type (containing Sp1 site and CCAAT-box) or mutant CCAAT-box DNA fragments were generated by PCR using indicated primer pairs and immobilized on streptavidin beads. After incubation with nuclear extracts, the DNA-bound Sp1 complexes were analyzed by gel electrophoresis followed by immunoblotting using an antibody to Sp1. Competition assay were performed in the presence of a 5-fold excess of unlabeled wild-type or mutant CCAAT-box DNA fragments. (F) 500 ng wild-type construct (-216/+32) or its mutants (Sp1MUT, and Sp1+CCAAT MUT) was cotransfected with or without 200 ng Sp1 expression vector into HT1080 cells. Firefly luciferase activity was normalized to renilla luciferase activity. Fold activation values were calculated by setting without Sp1 expression vector promoter activity to 1. Mean values (±) represent from three independent experiments. (G) Co-transfection with Sp1 expression vector, overexpression Sp1 was shown in HT1080 cells. *, $p<0.05$

**A**

```
                          -20       -10        +1       +10       +20       +30
Wild type:       5′  ACATGTGAGATAGTCACAGTTTTCCAGAGATCACAACAAGATCTAACCAGTCGCGCG  3′

MUT1                                            AgttC

MUT2                                         CctcGA

MUT3 (EtsA MUT)                           TTaaA

MUT4                                  CAaAaTTT

MUT5 (GATA MUT)               ATtttTtA

MUT6 (E-box MUT)       ATtTtcGA

MUT7                   ATtTtcGAaAtTtAaAaTTT
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**B**

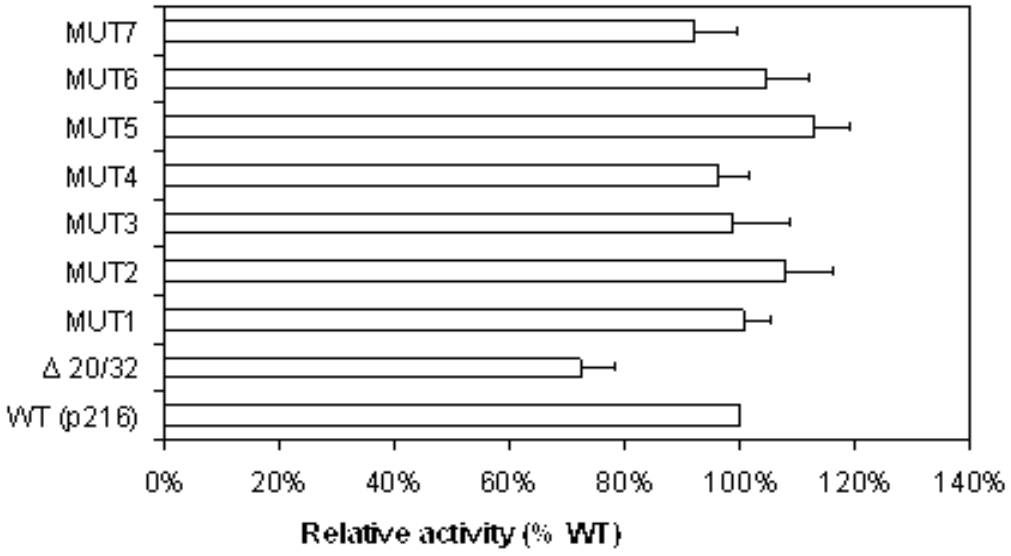


Figure 3.3 See overfeaf for legend

**Fig. 3.3**. Functional characterization of the putative DPE in the human TBX2 gene. (A) Nucleotide sequence of the region in which site-specific mutations were introduced in the TBX2 gene. The mutated nucleotides are indicated with lowercase letters, and the putative DPE is underlined. (B) 500 ng of the proximal TBX2 promoter (-216/+32) construct, its mutant or the deletion proximal promoter (-216/+19) construct was co-transfected with 50 ng pRL-TK into HT1080 cells. Firefly luciferase activity was normalized to renilla luciferase activity. The basal activity of the -216/+32 construct was set at 100%. Mean values (±) represent from three independent experiments.

### 3.3. Discussion

This study reports on the successful cloning of the 5'-regulatory region of the human TBX2 gene from the WI-38 fibroblasts and the identification of cis-elements essential for its basal transcriptional activity. While no consensus TATA box was found, a Sp1 and an inverted CCAAT box element were identified that are essential for basal promoter activity. This is in keeping with previous studies that have demonstrated that proximal Sp1 and CCAAT box elements play a critical role in activating transcriptional initiation in TATA-less promoters (Samson and Wong, 2002; Mantovani, 1999). Furthermore, inverted CCAAT boxes are more frequently found in TATA-less promoters (Mantovani, 1998). Our data showed that individual mutations of the Sp1 and CCAAT box motifs caused 6.7-fold and 4.6-fold decrease in transcriptional activity of the TBX2 proximal promoter respectively. We further demonstrated that Sp1 and NF-Y specifically bind to putative Sp1 and CCAAT box elements respectively and are constitutively bound to the proximal TBX2 promoter in vivo.

The physical interaction between Sp1 and NF-Y was previously reported to result in transcriptional activation of several genes (Liang et al., 2001; Yamada et al., 2000). The juxtaposition of Sp1 and CCAAT box elements in the TBX2 promoter therefore led us to further explore the possibility that Sp1 co-operates with NF-Y in regulating transcription of the TBX2 gene. DAI analysis showed no significant difference in the binding of the Sp1 protein to a wild type TBX2 promoter fragment and a TBX2 promoter fragment with a mutation within the CCAAT box. Although we cannot rule out the possibility that a potential physical interaction may exist between Sp1 and NF-Y, the findings of our functional analysis imply that physical and functional interaction between Sp1 and NF-Y may not be required to maintain optimum basal activity of the TBX2 promoter.

Both Sp1 and NF-Y have been shown to associate with TATA box binding protein (TBP) and several TB-associated factors (TAFS) (Bellorini et al., 1997; Frontini et al., 2002; Emili et al., 1994; Chiang et al., 1995; Tanese et al., 1996) which are believed to be critical in transcription initiation. To our surprise, overexpression of Sp1 failed to increase wild type TBX2 promoter activity, but led to an increased activity (2-3-fold) of the TBX2 promoter carrying a mutation in the CCAAT box motif. Taken together this suggests that while NF-Y and Sp1 have overlapping roles in transcription initiation they also have distinct roles which complement one another. Thus, a dynamic balance in their levels

appears to be important for maintaining optimum basal activity of the TBX2 promoter. This may explain why mutation of both Sp1 and CCAAT box motifs resulted in a stronger (13.5-fold) decrease in luciferase activity compared with the combined effect seen when they were individually mutated (11.3-fold). This difference was regarded as potentially important because it was observed in every experiment performed. Our findings thus reveal possible new insights into the regulation of transcription by Sp1 which may depend on the context of the promoter.

Core promoters comprise DNA sequence motifs within -40 to +40 nt relative to the transcriptional start site. A key role of the core promoter is to direct the initiation of transcription and the TATA box, the Initiator (Inr) and DPE are well described core promoter elements. The DPE consensus sequence is A/G-G-A/T-C/T-G/A/C and is generally located at around +28 to +34. The DPE is most commonly found in TATA box-less promoters (Smale and Kadonaga, 2003). Here, we show that a potential DPE-like motif is located between +26 and +32 relative to the transcriptional start-site in the proximal TBX2 promoter and deletion of this motif caused a significant decrease (25%) in promoter activity. Although previous studies have shown that the DPE together with the Inr participates in regulating transcription initiation (Burke and Kadonaga, 2006), we did not find any other potential motif which significantly altered basal promoter activity around the transcription start site (from -30 to + 10). Although the DPE has been shown to be present in human genes, most work reporting on its involvement in transcriptional regulation has focused on Drosophila genes (Smale and Kadonaga, 2003). Whether the DPE identified in the TBX2 promoter is functional is worthy of further investigation and future work will be needed to determine whether general transcription factors are able to bind to it.

In mouse melanocytes and melanoma cells, the transcription factors Mitf and USF-1 have been shown to activate the mouse *Tbx2* promoter through binding an E-box element in the proximal promoter (Carreira et al., 2000). Our current data suggest that this E-box element is not essential for basal activity of the TBX2 proximal promoter. Gel shift analysis however showed that this E-box element was bound by nuclear factor(s) (see appendix 2). To date, a battery of transcription factors have been shown to recognize the E-box element. The complex mechanisms involved in the regulation of gene expression include competitive binding, protein-protein interaction and chromatin

remodeling (Bouchard et al., 2007; Baudino and Cleveland, 2001; Boyd et al., 1998). Taken together, this implicates a potential role for the E-box element in regulating tissue specific and inducible expression of the Tbx2 gene.

Having cloned the human TBX2 5'-regulatory region and having identified key cis-acting elements required for its basal transcription we next focused on the regulated expression of TBX2.

## 3.4. Materials and methods

### 3.4.1. Cloning of the 5'-flanking region of the human TBX2 gene

We have previously demonstrated high levels of TBX2 expression in normal human WI-38 lung fibroblasts (Teng et al., 2007). Based on the human genome sequence database from http://www.ensemble.org, we designed primers (Forward: 5'-GTAGGGATAACGGTCGCAGA-3'; Reverse: 5'-GCGCGACTGGTTAGATCTTG-3') to enable us to amplify the 5'-regulatory region of the TBX2 gene. Human genomic DNA isolated from WI-38 cells was used to clone the 5'-flanking region of the TBX2 gene by polymerase chain reaction (PCR) and the PCR product was cloned into the SmaI site of the pGEM-3Z vector. The orientations and the sequences of the inserts were confirmed by restriction enzyme digestion and sequencing.

### 3.4. 2. Plasmid constructs

The human TBX2 promoter luciferase reporters were generated by inserting the SacI(-1606)-HindIII(+32), MluI (-1081)-HindIII (+32), KpnI (-955)-HindIII (+32), SmaI (-365)-HindIII (+32), SspI (-216)-HindIII (+32), and SspI (-216)-BglII (+19) fragments of the TBX2 gene (Fig. 1A) into the appropriately cleaved luciferase reporter vector pGL3-basic (Promega, USA). The pRL-TK vector (Promega, USA) was used as an internal control reporter to test for transfection efficiency. The pCMV-Sp1 expression vector was obtained from Dr. Tjian (Department of Molecular & Cell biology, University of California, Berkeley) through Addgene.

Site-directed mutagenesis was carried out with the QuickChange kit from Stratagene following the manufacturer's instructions. All mutant constructs were generated using the proximal promoter construct (-216/+32) as a template. The oligonucleotides used for mutagenesis (only sense-strand presented and mutations indicated with lowercase letters) are the following:

Mut1: 5'-AGTCACAGTTTTCCAGAGAgttCGACAAGATCTAACCAGTCG C-3'
Mut2: 5'-GTGAGATAGTCACAGTTTTCCctcGATCACGACAAGATCTAACCAG-3'
Mut3: 5'-CATGTGAGATAGTCACAGTTTTaaAGAGATCACGACAAGATCTAAC-3'
Mut4: 5'-GCAGGACATGTGAGATAGTCAaAaTTTTCCAGAGATCACGACAAG-3'

Mut5: 5'-CTGGGCAGGACATGTGAGATttTtACAGTTTTCCAGAGATCACG-3'
Mut6: 5'-TCCGAGCTGGGCAGGACATtTtcGATAGTCACAGTTTTCCAGAG-3'
Mut7: 5'-CAGGACATtTtcGAaAtTtAaAaTTTTCCAGAGATCACGACAAGATCTAAC-3'
The mutated nucleotide sequences of all mutant constructs were verified by sequencing.

### 3.4.3. Cell culture

WI-38 human embryonic lung fibroblasts (ATCC CCL-75) and HT1080 human fibrosarcoma (ATCC CCL-120) cell lines were maintained in Dulbecco's-modified Eagle medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in an atmosphere of 5% $CO_2$.

### 3.4.4. Transfections and reporter assays

Cells were plated at 1.5 x $10^5$ cells/ml in six-well plates 1 day before transfection. Non-liposomal mediated gene transfer was performed using FuGENE®6 (Roche Applied Science) according to manufacturer's instructions with 1 µg of DNA consisting of the reporter construct, and/or the Sp1 expression vector and the internal control vector. Thirty hours after transfection, cells were analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay (Promega, USA) following manufacturer's instructions and quantified with a Luminoskan Ascent Luminometer (Thermo LabSystems, USA).

### 3.4.5. Electrophoretic mobility shift (EMSA) and DNA affinity immunoblotting (DAI) Assays

Nuclear extracts were prepared from WI-38 cells as previously described (Lee and Green, 1990). For EMSA, double-stranded oligonucleotides containing a Sp1 site or CCAAT-box were end-labeled with [γ-$^{32}$P]ATP using T4 polynuceotide kinase. The labeled probes were incubated with 5 µg nuclear extracts in binding buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol and 2 µg poly(dI-dC) ] in a final volume of 20 µl . For supershift analysis, antibodies for Sp1 or NF-YA (Santa Cruz Biotechnology) were preincubated with nuclear extracts in binding buffer before the addition of probes. The reactions were resolved on 5% native polyacrylamide gels in 0.5xTBE buffer at 4°C. Oligonucleotides used in this experiment

are as follows (only sense-strand presented and mutations indicated with lowercase letters):

Sp1 Wild type: 5'-CTGTCAAAACCCCGCCCCCGGAGACCC-3'

Sp1 Mut: 5'-CTGAGCTGTCAAAACCCaGaaCCCGGAGACCCACAATTG-3'

CCAAT-box Wild type: 5'-GAGACCCACAATTGGTCCAAAAAGCGTAA-3'

CCAAT-box Mut: 5'-CCGGAGACCCACAATTatTCCAAAAAGCGTAAAATCAGCAATC-3'

For DAI assays, biotinylated DNA probes were generated by PCR using primer pairs of 5'-biotin modification (Forward: 5'-TGGCCTGAGCTGTCAAAAC-3'; Reverse: 5'-GCGCGACTGGTTAGATCTTG-3') and immobilized on Dynabeads Streptavidin (Dynal Invitrogen) according to the manufacturer's instructions. For each reaction, 40 µg nuclear extract was incubated with 1 µg DNA probe in binding buffer (same as for EMSA) in final volume of 400 µl. The beads were extensively washed with binding buffer and then boiled in 25 µl of 2x loading buffer before SDS-polyacrylamide gel electrophoresis followed by immunoblotting.

### 3.4.6. Chromatin immunoprecipitation assay (ChIP)

ChIP assays were carried out as previously described (Prince et al., 2004). Briefly, WI-38 cells expressing endogenous TBX2 were fixed in 1% formaldehyde. The chromatin was extracted in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL, pH 8.1) with a proteinase inhibitor, sonicated to an average length of about 500 bp, and DNA-containing fractions were incubated overnight with antibodies to Sp1 or NF-YA and collected on protein G beads. Cross-linked products were reversed by heating at 65°C for 4 hours, and the immunoprecipitated DNA was purified by proteinase *K treatment.* The precipitated DNA was analyzed by PCR using TBX2 specific primer pairs (Forward: 5'-TGGCCTGAGCTGTCAAAAC-3'; Reverse: 5'- GCGCGACTGGTTAGATCTTG-3').

# CHAPTER 4

## PHOSPHORYLATION OF HISTONE H3 BY BOTH PKC AND MAPK SIGNALLING PLAYS A CRITICAL ROLE IN ACTIVATING THE EXPRESSION OF THE HUMAN TBX2 GENE

### 4.1. Introduction

The nucleosome is the basic building unit of chromatin and consists of a protein core of a histone octamer (H2A, H2B, H3 and H4) with 146 base pairs of DNA wound around it. N-terminal tails of histones protrude outside of the nucleosome and are thus readily subject to a variety of covalent modifications such as acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation. These modifications have been proposed to cause an alteration of chromatin configuration and/or form a recognition motif allowing access of relevant proteins of transcription to regulate gene expression (Berger, 2002; Jenuwein and Allis, 2001). For example, phosphorylation of histone H3 has been associated with transcriptional activation of c-jun and c-fos immediate-early (IE) response genes in response to growth factors and phorbol esters (Mahadevan et al., 1991). The list of genes identified that are associated with stimulus-induced phosphorylation of H3 is growing (Clayton and Mahadevan, 2003).

Protein Kinase C (PKC) represents an important multigene family of serine/threonine kinases that can be activated by various extracellular and intracellular signals and have been involved in broad and diverse functions in many physiological and pathological processes (Newton, 1997; Griner and Kazanietz, 2007). The PKC family comprises at least 12 isoforms that are divided into three subfamilies: conventional or classical PKCs (cPKCs; α, β 1/2 and γ), novel PKCs (nPKCs; δ, ε, η, and θ), and atypical PKCs (aPKCs; ζ and λ/ι). PKC is a major intracellular target of 12-O-tetradecanoylphorbol-13-acetate (TPA) and it is well established that in response to TPA, PKCs can activate the Ras/Raf/MEK/ERK MAPK pathway. There is also accumulating evidence suggesting that TPA can activate p38 and JNK in a cell-type dependent manner (López-Bergami et al., 2005; Mauro et al., 2002).

Given the important role that Tbx2 plays in embryonic development and carcinogenesis there is a need to understand the mechanism(s) regulating Tbx2 gene expression. However, while several signalling pathways, including FGF, BMP and Shh have been associated with the regulation of Tbx2 expression the details of this regulation remain unknown (Rowley et al., 2004; Suzuki et al., 2004; Nissim et al., 2007). One of the aims of the present study was therefore to investigate the regulation of Tbx2 gene expression by signalling pathways and the results demonstrate that TPA induces TBX2 gene expression in normal human lung fibroblasts in a PKC-dependent and MAKP-independent manner. However, we also show that p38 and Erk1/2 signalling are involved in regulating TBX2 gene expression in transformed human fibroblasts. We further reveal that TPA-induced phosphorylation of histone H3 plays a critical role in regulating TBX2 gene expression. Moreover, we provide evidence to link PKC to MSK1, a potential histone H3 kinase.

## 4.2. Results

### 4.2.1. Induction of TBX2 gene expression by TPA in human fibroblasts

We have previously shown that TBX2 is expressed at high levels in the normal human lung fibroblast cell line, WI-38, but that it is expressed at very low levels in WI-38 transformed cell lines, CT and SVWI-38 (Teng et al., 2007). To identify signalling pathways that regulate TBX2 expression we first examined the effect of TPA treatment of WI-38 cells on Tbx2 by western blot analysis. Our results show that TPA significantly induces Tbx2 expression in a time-dependent manner (Figure 4.1A). The up-regulation of Tbx2 can be observed from 4 hr to 24 hr, and maximally at 8 hr. Interestingly, TBX2 expression was strongly induced in the CT and SVWI-38 cells, which express very low levels of Tbx2, when they were treated with TPA for 8 hrs (Figure 4.1B).

In order to determine whether the above induction of Tbx2 expression by TPA is due to an increase in transcription, cells were treated with or without TPA for a period spanning 1 hr to 24hr and Tbx2 mRNA levels assessed by real-time PCR. Our results show that Tbx2 mRNA levels were up-regulated from 2hr with levels peaking at 8hr (Figure 4.1C) in WI-38 cells. The same increase in Tbx2 mRNA levels was observed when SVWI-38 and CT-1 cells were treated with TPA for 8 hr (Figure 4.1D). In response to TPA, Tbx2 mRNA levels therefore closely correlate with levels of Tbx2 protein which suggest that TPA induces Tbx2 expression at a transcriptional level.

### 4.2.2. Induction of TBX2 gene expression by TPA in WI-38 cells is PKC-dependent

To investigate the pathway(s) by which TPA up-regulates TBX2 gene expression in WI-38 cells, we first examined the phosphorylation of MAPKs by western blot analyses. Figure 4.2A shows that 1hr to 8 hr of TPA treatment leads to an increase in Erk1/2 phosphorylation, a marginal increase in phosphorylated p38 at only 4 hr treatment and undetectable changes in JNK phosphorylation.

To assess the involvement of the above signalling molecules in TPA-induced expression of TBX2, we next pretreated WI38 cells with inhibitors to PKC (GF109203X), Erk1/2

(PD98059), p38 (SB203580), and JNK (SP600125) before TPA treatment and then analysed TBX2 expression by western blotting. As shown in Figure 4.2B, while both PD98059 and SB203580 have no effect on the induction of Tbx2 expression at 8 hr of TPA treatment, SP600125 block TPA-induced Tbx2 expression at this time point. Similar results were also obtained at 4hr of TPA treatment (see appendix 3). It is interesting to note that GF109203 repress both TPA-induced and basal TBX2 expression. This finding suggests that constitutive activity of PKC may be essential for TBX2 expression in WI-38 cells.

To determine the blocking efficiency and specificity of the above inhibitors, their effect on phosphorylation of MAPKs were examined. Our results (Figure 4.2C, D) show that PD98059 and SB20358 can sufficiently reduce phosphorylation of Erk1/2 and p38 respectively. Surprisingly, although TPA was not shown to enhance JNK phosphorylation, TPA-mediated TBX2 expression was repressed by SP600125

### 4.2.3. Regulation of Tbx2 gene expression by MAPK signalling is cell type-dependent

To explore this possibility with regard to TPA-mediated Tbx2 expression the above experiment was performed in CT-1 and SVWI-38 cells. The results shown in Figure 4.3A and 4.3B reveal that PD98059 reduced the effect of TPA on TBX2 expression in both SVWI-38, and CT-1 cells respectively and this corresponded with a block in Erk phosphorylation (Figure 4.3C and 3D). Interestingly, a similar effect is seen when CT-1, but not SVWI-38, cells were pretreated with SB203580.

### 4.2.4. TPA and AP1 does not activate a Tbx2 gene reporter construct

It is well-documented that TPA induces the expression of numerous genes through up-regulation of AP1 and we therefore examined the expression of c-Jun and JunB in WI-38, SVWI-38 and CT-1 cells treated with or without TPA by western blot analyses. Results show that TPA increases both c-Jun and JunB protein levels in a time-dependent manner which correlates with an up-regulation of TBX2 in all three cell lines tested (Figure 4.4 A, B).

To determine whether AP-1 can indeed regulate the TBX2 promoter we searched the 3000bp 5'-flanking regulatory sequence of the human TBX2 gene which was obtained from the human genome sequence database (http://www.ensemble.org), for potential cis-acting elements using the TFSEARCH program (www.cbrc.jp/research/db/TFSEARCH.html). A putative AP-1 site was found at -1069 relative to the transcriptional start site (data not shown) and we thus performed luciferase assays using a reporter construct driven by a 1638bp sequence of the TBX2 gene containing this putative site (see Chapter 3). TPA failed to stimulate transcriptional activity of the human TBX2 promoter in SVWI-38 and HT1080 cells (Figure 4.4C), and over-expression of c-Jun or JunB did not significantly increase Tbx2 promoter activity in HT1080 cells (Figure 4.4D). These findings led us to hypothesize that either distal elements not present in the 1638bp Tbx2 regulatory fragment may be mediating the effects of TPA or that a chromatin remodeling mechanism may be involved in transcriptional regulation of TBX2 gene expression by TPA.

### 4.2.5. Phosphorylation of histone H3-Ser10 is required for binding of Sp1 to the TBX2 gene in WI-38 cells

Several lines of evidence suggest that translational modification of histone H3 leads to the activation of genes in a Sp1 site dependent manner (Han et al., 2001; Tone et al., 2007; Takakura et al 2001; Schuettengruber et al., 2003). Furthermore, TPA-inducible histone H3 phosphorylation has been correlated with transcriptional activation of several immediate-early (IE) genes such as c-Fos and c-Jun (Clayton and Mahadevan 2003). We have previously demonstrated that the basal transcription of the human TBX2 gene is dependent on a Sp1 site in its proximal promoter (see chapter 3). This led us to ask the question as to whether TPA induces TBX2 expression through phosphorylating histone H3, hence recruiting Sp1 to the Sp1 site identified above. Western blot analyses were therefore performed to examine TPA-inducible phosphorylation of histone H3 in WI-38 cells. Our results show that TPA treatment results in increased levels of phosphorylated histone H3-Ser10 from 1hr and is sustained up to 8hrs of treatment (Figure 4.5A).

We next examined whether TPA induces histone H3-Ser10 phosphorylation at the TBX2 promoter using ChiP assays. Primers spanning the proximal promoter of TBX2 (Figure 4.5B) were used for PCR amplification of immunoprecipitated DNA fragments and

quantitative analysis was performed by real-time PCR. Compared to untreated cells, an approximately two fold increase in histone H3-Ser10 phosphorylation was obtained at the TBX2 promoter in WI-38 cells treated with TPA for 8hr (Figure 4.5C, D). To explore the possibility that TPA enhances Sp1 binding to the TBX2 promoter, the same primers shown in Figure 4.5B were used to amplify DNA fragments immunoprecipitated with an antibody specific to Sp1. A significant increase of Sp1 was observed at the TBX2 promoter in WI-38 cells treated with TPA (Figure 4.5C, D).

### 4.2.6. PKC-dependent phosphorylation of histone H3-Ser10 correlated with activation of MSK1

Whereas several kinases, including MSK1/2 (Thomson et al. 1999; Soloaga et al., 2003), ribosomal S6 kinase 2 (RSK2) (Sassone-Corsi et al., 1999), PKA (Salvador et al., 2001), PKC (Huang et al., 2004), and IκB kinase-α (IKK-α) (Park et al., 2006) have been proposed to phosphorylate histone H3 in response to various stimuli, current evidence suggests that MSK1 and 2 are the primary kinases involved (Clayton and Mahadevan 2003). We therefore investigated whether MSK1 was activated in WI-38 cells treated with TPA by reprobing the western blot shown in Figure 4.5A with an antibody to phosphorylated MSK1. An increase in MSK1 phosphorylation was evident from 1hr and persisted over a period of 24 hr of TPA treatment (Figure 4.5A). To identify the signalling pathway implicated in this phosphorylation, the effect of PKC (GF109203X), Erk (PD98059), p38 (SB20358) and JNK (SP600125) inhibitors were included in experiments in which WI-38 (Figure 4.6A), SVWI-38 and CT-1 cells (Figure 4.6B) were treated with TPA for 8hr. The results reveal that whereas both TPA-mediated phosphorylation of MSK1 and histone H3-Ser10 were abolished by GF109203X, no significant effect was observed for the PD98059 and SB20358 inhibitors in WI38 cell (Figure 4.6A). These findings suggest a functional link between PKC and MSK1 activation during TPA-mediated phosphorylation of histone H3-Ser10.

Interestingly, while both the PD98059 and SB20358 inhibitors led to a decrease in MSK1 phosphorylation in CT-1 cells treated for 8hr with TPA, we failed to detect alterations in global phosphorylation of histone H3-Ser10 under the same conditions (Figure 4.5B). Furthermore, PD98059 did not significantly affect both phosphorylation of MSK1 and

global phosphorylation of histone H3-Ser10 in SVWI-38 cells treated with TPA for 8hr (Figure 4.6B).

### 4.2.7. SP600125 reduces Phosphorylation of histone H3-Ser10 in a MSK1-dependent manner

Results shown in Figures 4.2A, C, D and 4.3C, D indicated that TPA did not significantly activate JNK in the three fibroblast cell lines used in this study. However, SP600125 was shown to block TPA-induced expression of TBX2 in all three these cell lines and this paralleled with the reduction of histone H3-Ser10 phosphorylation as well as MSK1 phosphorylation seen (Figure 4.6.). Interestingly, SP600125 has recently been identified to act as an inhibitor of histone H3-Ser10 phosphorylation through an unknown mechanism (Huang et al., 2007). Our findings suggest that SP600125 may repress phosphorylation of histone H3-Ser10 through blocking MSK1 activation. Furthermore, we also provide evidence to support a critical role of histone H3-Ser10 phosphorylation in activating the expression of the human TBX2 gene.

### 4.2.8. Effect of TPA on Sp1 expression and DNA binding activity

We have clearly shown here that TPA-induced phosphorylation of histone H3-Ser10 was associated with recruitment of Sp1 to a Sp1 site in the human TBX2 gene. However, several lines of evidence have also shown that TPA-mediated expression of certain genes is associated with increased levels of total and/or phosphorylated Sp1 (Samson and Wong, 2002). To test whether the TPA-mediated increase in TBX2 gene expression is also due to the latter mechanism involving increased levels of Sp1 we next examined the expression pattern of Sp1 in WI-38 cells treated with or without TPA. Western blotting reveals that there is no significant change in total (Figure 4.7A) and nuclear (Figure 7B) Sp1 protein levels at time points tested and thus excluded this possibility. In addition, Sp1 protein levels were not significantly influenced in the presence of the PKC, JNK, p38 and Erk inhibitors (4.7 C).

To further explore whether there was an effect of TPA on nuclear factors binding to the Sp1 site in the proximal TBX2 promoter, DAI assays were carried out. Our results show that while this site was specifically bound by Sp1 (Figure 4.7D right) TPA treatment did

not enhance Sp1 binding to this site (Figure 4.7D left). However, experiments in which unlabelled probe was included to compete with labeled probe indicated that nuclear factors from TPA-treated cells bound to the Sp1 site more stably compared to those from cells not treated with TPA (Figure 4.7D).

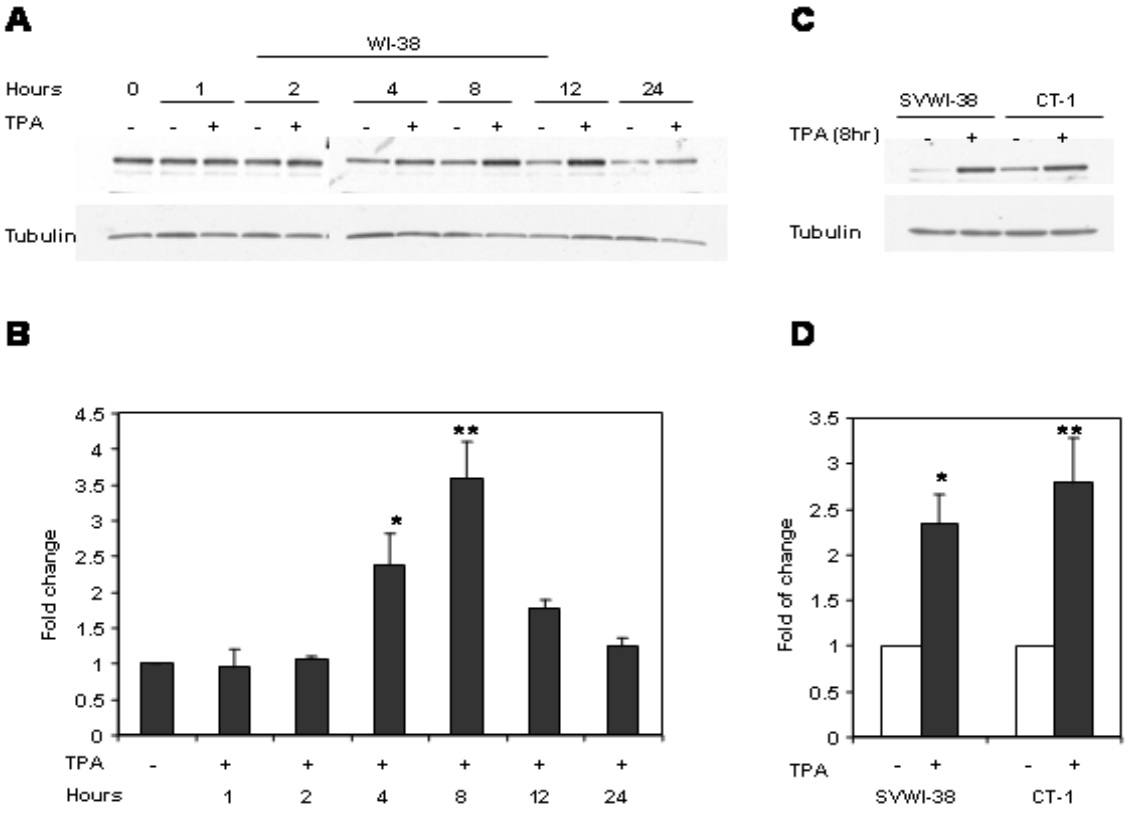


**Figure 4.1.** Induction of TBX2 expression by TPA in human fibroblasts. WI-38 (A and B) and SVWI-38 and CT-1 (C and D) cells were treated with either vehicle (control) or TPA (100 nM) for the indicated times. Whole cell lysates and total RNA were harvested and subjected to western blot (A and C) or real-time PCR (B and D) analyses, respectively. * $p<0.05$, ** $p< 0.01$.

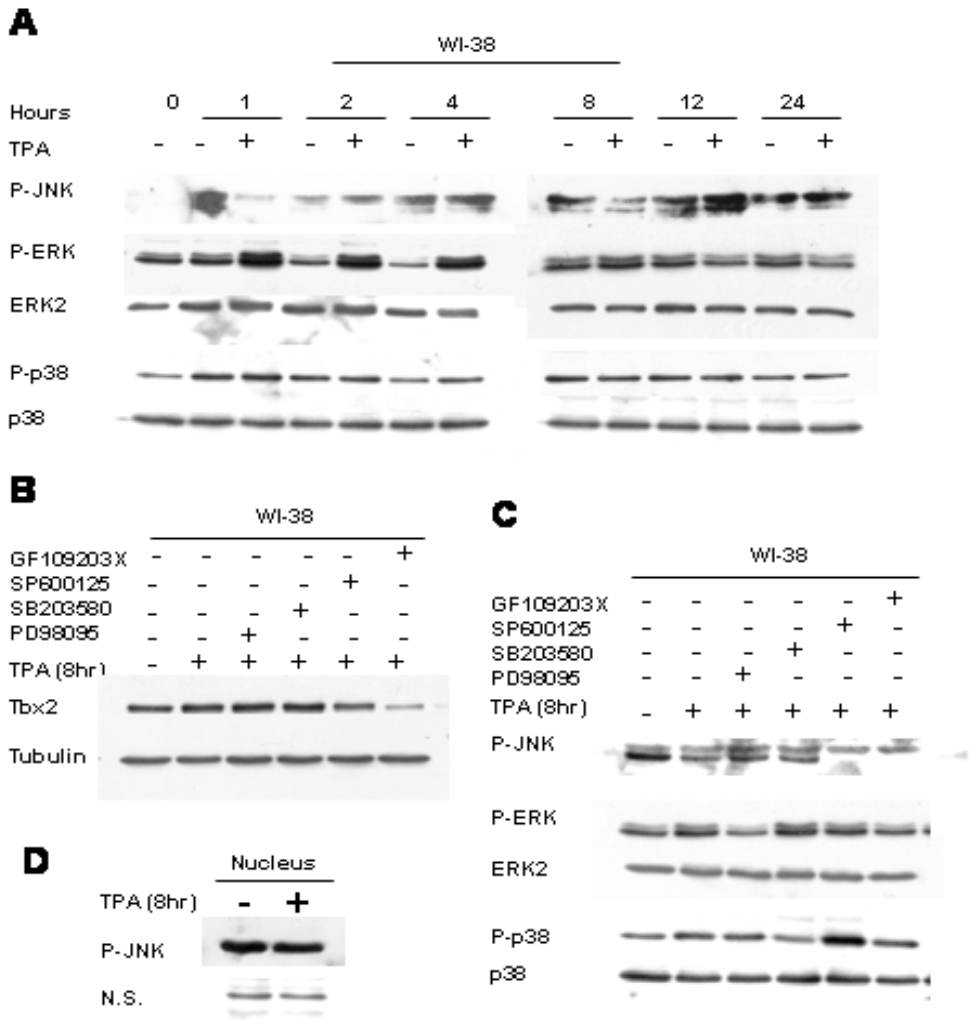


**Figure 4.2.** TPA stimulates TBX2 gene expression in a PKC-dependent manner in WI-38 cells. (A) WI-38 cells were treated with either vehicle (control) or TPA (100 nM) for the indicated times. Whole cell lysates were harvested and subjected to western blotting to detect phosphorylated JNK, p38, and Erk1/2. Total Erk2 or total p38 were detected as loading controls. (B and C) WI-38 cells were pretreated with vehicle or 20 μM GF109203X, 25 μM PD 98095, 20 mM SP600125 and 10 μM SB203580 for 1 hr and then treated with TPA (100 nM) for 8 hr. Western blots were carried out to detect levels of Tbx2 (B) and phosphorylated JNK, p38 and Erk1/2 (C). Total Erk2 or total p38 or α-tubulin was detected as loading control. (D) WI-38 cells were treated with vehicle or TPA for 8 hr and nuclear proteins were prepared and subjected to western blotting to detect phosphorylated JNK.

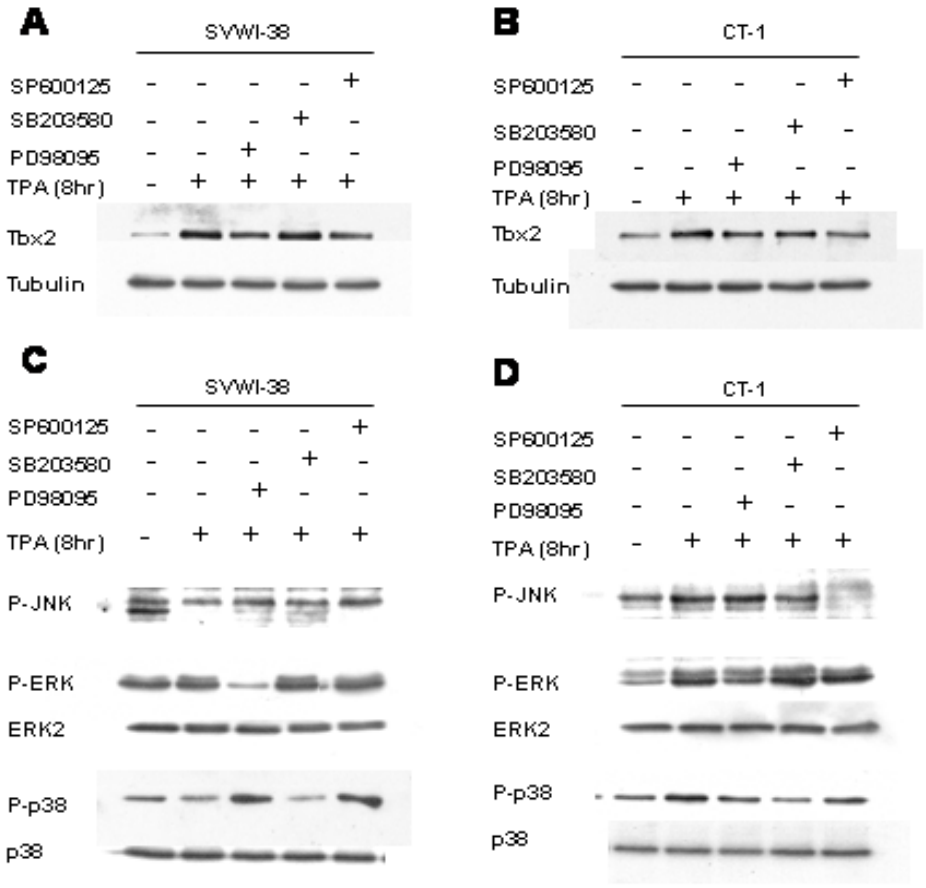




**Figure 4.3.** Erk and/or p38 signalling are involved in the regulation of TBX2 expression by TPA in SVWI-38 and CT-1 cells. SVWI-38 (A and C) and CT-1 (B and D) cells were pretreated with vehicle or 20 μM GF109203X, 25 μM PD 98095, 20Mm SP600125 or 10 μM SB203580 for 1 hr and then treated with TPA (100 nM) for 8 hr. Western blots were carried out to detect Tbx2 (A and B) and phosphorylated JNK, p38 and Erk1/2 (C and D). Total Erk2 or total p38 or α-tubulin was detected as loading control.

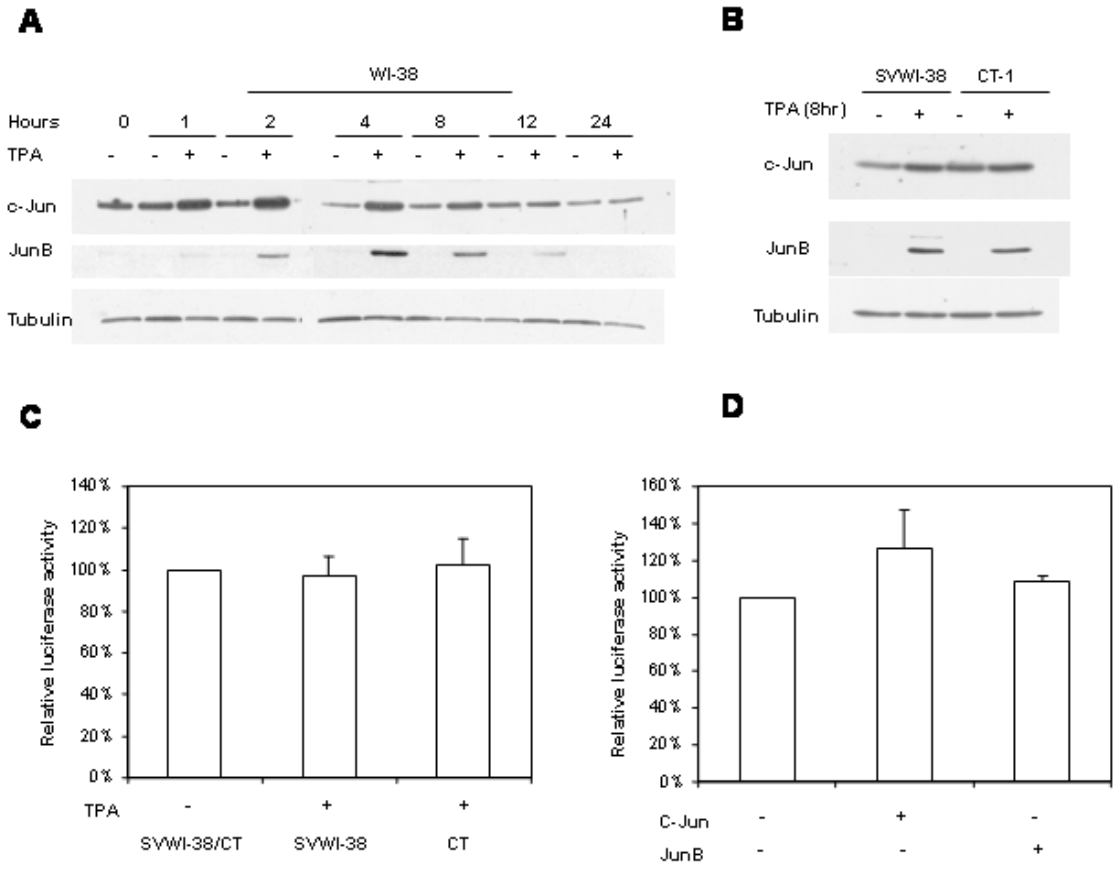


**Figure 4.4.** TPA, c-Jun and JunB do not affect human TBX2 promoter activity. (A and B) TPA stimulated expression of c-Jun and JunB in human fibroblasts. WI-38, SVWI-38 and CT-1 cells were treated with either vehicle (control) or TPA (100 nM) for the indicated times. Whole cell lysates were harvested and subjected to western blot analysis with the indicated antibodies. (C) The TBX2 promoter-luciferase reporter (500 ng) was co-transfected into SVWI-38 and CT-1 cells with the internal control pRL-TK (50 ng) in the presence or absence of TPA (100 nM for 12 hr). Luciferase activity was measured 30 hr post-transfection and normalized to renilla luciferase activity. Activation fold values were calculated by setting untreated promoter activity to 1. Mean values (±) were calculated from three independent experiments. (D) The TBX2 promoter-luciferase reporter (500ng) was co-transfected into HT1080 cells either with the empty pCMV (200 ng) vector or c-Jun or JunB (200 ng) expression vectors and luciferase activity determined as in (C) above. Activation fold values were calculated by setting the effect of empty pCMV vector on TBX2 promoter activity to 1. Mean values (±) were calculated from three independent experiments.

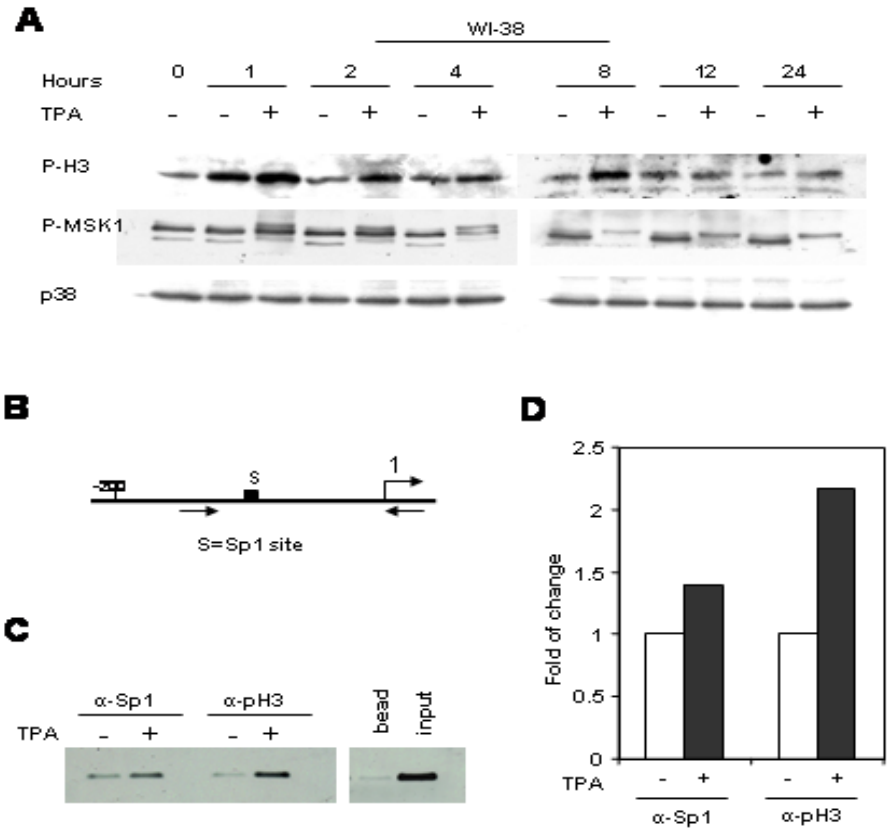


**Figure 4.5.** Induction of TBX2 expression by TPA in WI-38 cells is associated with recruitment of Sp1 to the TBX2 promoter and histone H3-Ser10 phosphorylation (A) TPA stimulated increase of global histone H3-Ser10 phosphorylation and activation of MSK1. WI-38 cells were treated with either vehicle (control) or TPA (100 nM) for the indicated times. Whole cell lysates were harvested and subjected to western blot analysis with the indicated antibodies. To enable the detection of phosphorylated MSK1, proteins were separated on 6% SDS-PAGE and where more than one band is present, the upper band represents the phosphorylated MSK1. (B) Schematic illustration of the region of TBX2 proximal promoter showing the putative Sp1 site (boxed). Arrows represent primer pairs used for PCR. (C) WI-38 cells were treated with either vehicle (control) or TPA (100 nM) for 8 hr and Chip assays were performed with antibodies against Sp1 or phospho-H3. Co-immunoprecipitated DNA was assayed by PCR with primer pairs indicated in (A) above. Inputs and no antibody are positive and negative controls respectively. (D) Co-immunoprecipitated DNA was assayed by real-time PCR and was normalized to input. Fold change values were calculated by setting untreated samples to 1.

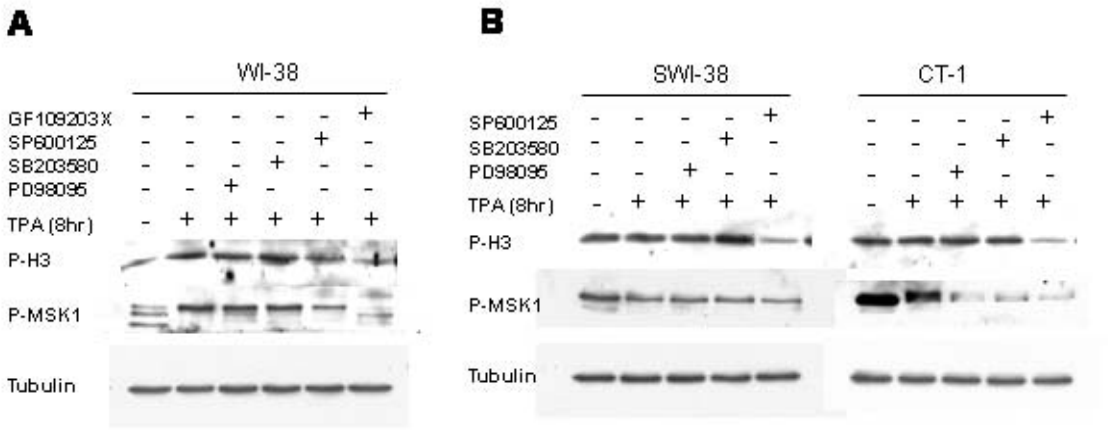


**Figure 4.6.** TPA activates MSK1 which correlates with phosphorylation of histone H3-Ser10 in a PKC-dependent manner. (A and B) WI-38, SVWI-38 and CT-1 cells were pretreated with vehicle or 20 μM GF109203X, 25 μM PD 98095, 20 Mm SP600125 or 10 μM SB203580 for 1 hr and then treated with TPA (100 nM) for 8 hr. Western blots were carried out to detect phosphorylated histone H3-Ser10 and MSK1 and tubulin was included as a loading control. To enable the detection of phosphorylated MSK1, proteins were separated on 6% SDS-PAGE and where more than one band is present, the upper band represents the phosphorylated MSK1.

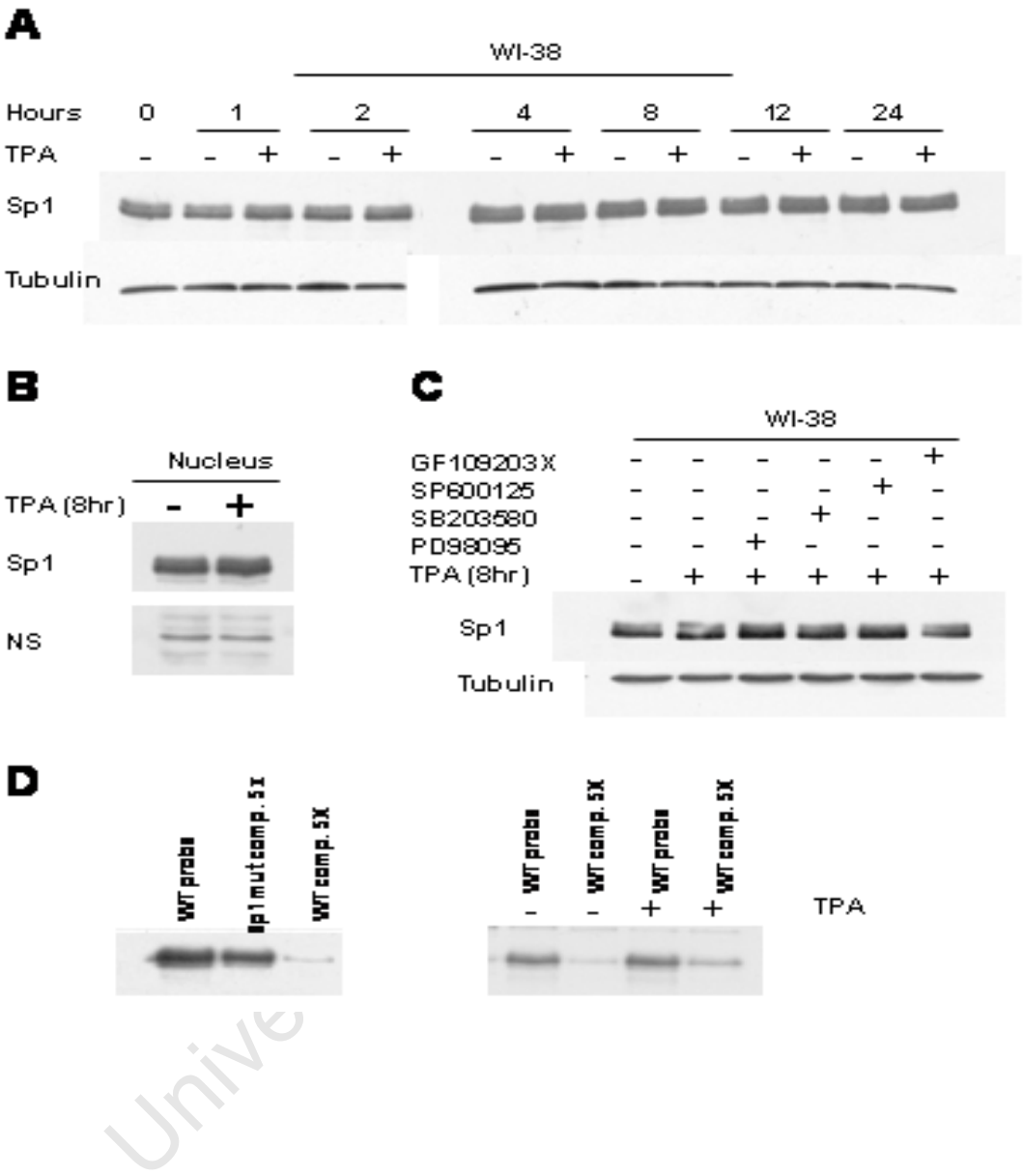


**Figure 4.7.** TPA has no effect on Sp1 expression but stabilizes its binding to the TBX2 promoter. (A) WI-38 cells were treated with either vehicle (control) or TPA (100 nM) for the indicated times and cell lysates subjected to western blot analysis to detect Sp1. (B) As for (A) above but cells were pretreated with vehicle or 20 μM GF109203X, 25 μM PD 98095, 20 Mm SP600125 or 10 μM SB203580 for 1 hr prior to TPA treatment (8 hr). (C) As for (A) above but nuclei were prepared for WI-38 cells treated with either vehicle (control) or TPA (100 nM) for 8 hr. (D) For DAI analysis, biotinylated DNA fragments were generated by PCR using indicated primer pairs (Figure 4.5B) and immobilized on streptavidin beads. After incubation with nuclear extracts (40 μg), the DNA-bound Sp1 complexes were analyzed by gel electrophoresis followed by immunoblotting using an antibody for Sp1. Competition assays were performed in the presence of a 5-fold excess of unlabeled wild-type or mutant CCAAT-box DNA fragments.

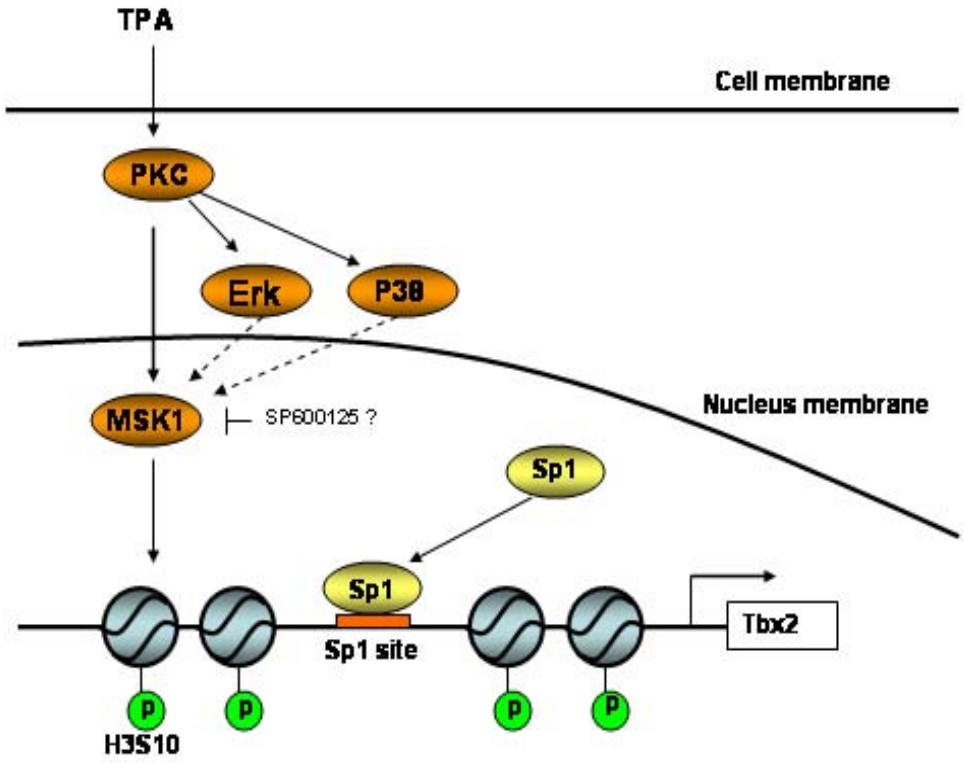


**Figure 8.** A schematic representation of proposed model. Phosphorylation of histone H3 leads to chromatin remodeling, hence recruitment of Sp1 to TBX2 promoter which results in the activation TBX2 gene transcription. In this process, TPA can activate the histone H3 kinase MSK1 through mainly PKC, but also through a PKC-dependent Erk and p38 MAPK depending on the cell-type.

## 4.3. Discussion

The mechanisms involved in the transcriptional regulation of the Tbx2 gene are still poorly understood. In view of the detrimental consequences resulting from altered levels of T-box proteins, as seen in both developmental disorders and in certain cancers, the need to identify such mechanisms is important. Here we demonstrate for the first time that TPA treatment of normal and transformed human fibroblasts leads to a time-dependent increase in Tbx2 mRNA and protein levels and we provide compelling evidence that this results from TPA regulating TBX2 transcription through a process involving phosphorylation of histone H3.

It is well established that phosphorylation of histone H3 plays a critical role during transcriptional activation of IE genes in response to various stimuli. Phosphorylation of histone H3 has also been linked to transcriptional activation of several relative late response genes (Clayton and Mahadevan 2003; Ge et al., 2006). Here, we show that TPA increases both histone H3-Ser10 phosphorylation and Sp1 recruitment to a Sp1 site in the TBX2 proximal promoter which correlates with the induction of TBX2 expression. This is in keeping with our previously published data which identified this Sp1 site to be essential in maintaining basal activity of a human TBX2 promoter (see chapter 3). Moreover, blocking histone H3-Ser10 phosphorylation is associated with abolishing Tbx2 induction. These findings suggest TPA activates transcription of TBX2 through phosphorylation of histone H3 and recruitment of Sp1 to the Sp1 site in the TBX2 proximal promoter.

Phosphorylation of H3 has also been shown to be necessary for chromosome condensation and chromosome segregation during mitosis (Gurley et al., 1978; Hendzel et al., 1997).To date, it is well-known that stimulus-mediated phosphorylation of histone H3 is transient and involves only a small fraction of histone H3 which is distinct from phosphorylation of the majority of H3 observed during mitosis (Clayton and Mahadevan 2003; Johansen, 2006). Stimulus-mediated phosphorylation of histone H3 may therefore not be observed at a global level. Indeed, a reduced phosphorylation of global histone H3-Ser10 was found in some instances (Huang et al., 2004; Nowak and Corces 2000). This may explain why we did not find an increase in global histone H3-Ser10

phosphorylation in two transformed fibroblast cell lines and at a later time point in normal fibroblasts.

MSK1 has been well characterized as a histone H3 kinase (Thomson et al. 1999; Soloaga et al., 2003). Mitogen- and stress- induced phosphorylation of histone H3 has also been demonstrated to be mediated by the Erk and/or p38 MAPK cascades in many different systems (Clayton and Mahadevan 2003). PKC has been proposed as a histone H3 kinase responsible for histone H3-Ser10 phosphorylation at the LDL receptor promoter in response to TPA in human hepatoma HepG2 cells (Huang et al., 2004). Here we show that TPA activates MSK1 which is associated with phosphorylation of histone H3 in a PKC-dependent and MAPK- independent manner in WI-38 cells. Although we can not rule out the possibility that PKC may also directly phosphorylate histone H3-Ser10, our findings clearly suggest that MSK1 is a potential downstream target of PKC and is linked to phosphorylation of histone H3.

Although we did not detect activation of JNK by TPA in our system, it is intriguing to note that the JNK inhibitor, SP600125, resulted in an apparent repression of TBX2 expression which was associated with a reduction of global phosphorylation of histone H3. Consistent with our finding, SP600125 has been shown to reduce global histone H3-Ser10 phosphorylation in a JNK MAPK independent manner in human hepatocellular HepG2 cells (Huang et al., 2006). However, our results also indicated that SP600125 inhibits MSK1 activity. Since SP600125 has been shown to be capable of inhibiting a variety of kinases (Bain et al., 2003), it is possible that it can act as an inhibitor of MSK1 and thus block phosphorylation of histone H3. This possibility is worthy of further investigation.

Although other studies have suggested that TPA leads to an increase in Sp1 levels in several systems (Noe et al., 2001; D'Angelo et al., 1996; Pornttadavity et al., 2001), our data here did not show that TPA can significantly affect Sp1 protein level in all types of fibroblast tested. However, TPA was shown to significantly enhance stability of nuclear factor binding to Sp1 site in TBX2 promoter. TPA-induced phosphorylation of Sp1 has been shown to enhance Sp1 binding and transcription activity in human HepG2 cells (Zhang X-L et al., 2000), and phosphorylation and activation of Sp1 have also been shown to be in PKC and MAPKs-dependent manner (Samson and Wong, 2002; Chu

and Ferro, 2005). Thus, whether Sp1 phosphorylation is also implicated in TPA-mediated transcription activation of the TBX2 gene remains to be elucidated. Furthermore, we show that while TPA upregulates c-Jun/AP1 and JunB/AP1 expression which correlates well with up-regulation of Tbx2, TPA and c-Jun/JunB failed to activate a reporter gene driven by a 5'- regulatory region of the TBX2 gene. These findings provide indirect support for the possibility that TPA-mediated phosphorylation of histone H3-Ser10 plays a critical role in transcriptional activation of the TBX2 gene.

In conclusion, we show that in response to TPA, phosphorylation of histone H3 leads to the recruitment of Sp1 to a Sp1 site in the proximal TBX2 gene promoter which is critical for the induction of TBX2 gene expression. Furthermore, we demonstrate that this is mediated by PKC and involves MSK1. Interestingly, while MSK1 seems to be directly activated by PKC in normal fibroblasts, its activation appears to occur via Erk or p38 signalling pathways in transformed fibroblasts (Figure 8). Our findings give insight into the molecular mechanism(s) that regulate TBX2 gene expression.

## 4.4. Materials and methods

### 4.4.1. Cell culture and reagents

WI-38 (ATCC CCL-75) normal human embryonic lung fibroblasts, their in vitro transformed counterparts WI-38 CT-1 (referred to as CT-1; Namba et al., 1980) and SVWI-38 cell lines were maintained in Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in an atmosphere of 5% $CO_2$. For TPA treatment, 100 nM TPA was used (Sigma). The PKC inhibitor GF109203 (20 µM), MEK-1 inhibitor PD98095 (25 µM), p38 inhibitor SB203580 (10 µM) and JNK inhibitor SP600125 (20 µM) (Calbiochem) were added 1 hr prior to treatment with TPA.

### 4.4.2. Plasmids, Transfections and reporter assays

The human TBX2 promoter luciferase reporter construct was generated by inserting the SacI(-1606)-Hind III(+32) fragment of the human TBX2 gene into the appropriately cleaved luciferase reporter vector pGL3-basic (Promega, USA). The pRL-TK vector (Promega, USA) was used as an internal control reporter to test for transfection efficiency. The pCMV-c-Jun and pCMV-JunB expression vectors were kindly provided by Dr Michael Birrer (National Institutes of Health, USA). Cells were plated at 1.5 x $10^5$ cells/ml in six-well plates 1 day before transfection. Non-liposomal mediated gene transfer was performed using FuGENE®6 (Roche Applied Science) according to manufacturer's instructions using 1 µg of DNA consisting of the reporter construct, expression vectors and the internal control vector. Thirty hours after transfection, cells were analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay (Promega, USA) following manufacturer's instructions and quantified with a Luminoskan Ascent Luminometer (Thermo LabSystems, USA).

### 4.4.3. Western blotting

Western blot assays were carried as described previously (Teng et al., 2007). Proteins were resolved on 8-12% SDS-polyacrylamide gels, as required and transferred to Hybond ECL (Amersham Biosciences, USA). The membranes were probed with

appropriate primary antibodies and detected using peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:5000) and visualised by ECL (Pierce, USA). The primary antibodies used were: mouse monoclonal anti-Tbx2 62-2 antibody (1:2000), rabbit polyclonal anti-phospho-H3 (1:2000, Upstate Biotechnology), anti-phospho-p38 (1:1000), anti-p38 (1:5000), anti-phospho-MSK1 (1:1000), anti-phospho-Erk1/2 (1:1000), anti-phospho-JNK (1:1000) (Cell Signaling Technology Inc., Beverly, MA), rabbit polyclonal anti-c-Jun, JunB, Erk2, Sp1 (1:500) and mouse monoclonal alpha-tubulin (1:500) from Santa Cruz Biotechnology, CA, USA.

#### 4.4.4. Real time RT PCR

Total RNA was extracted from cultured cells and quantitative RT PCR assays were carried out using the LightCycler as previously described (Teng et al., 2007). Primers used for RT-PCR were human TBX2: (forward) 5'- ATGGGCATGGGTCACCTACT-3'; (reverse) 5'- GGTGTAGGGGTATTTTAAGA -3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-GAAGGCTGGGGCTCATTT-3'; reverse 5'-CAGGAGGCATTGCTGATGAT-3' 3 PCR conditions were as follows: 95°C for 10 min; and 45 cycles of 95°C for 45 seconds (denaturation), 59°C for 20 seconds (annealing), and 72°C for 20 seconds (extension). The PCR products of TBX2 were cloned into the pGEM-T easy vector (Promega, USA.) and verified by sequencing.

#### 4.4.5. DNA affinity immunoblotting (DAI)

Nuclear extracts were prepared from WI-38 cells as previously described (Lee and Green 1990). For DAI assays, biotinylated DNA probes were generated by PCR using primer pairs that were synthesized and 5' end-labeled with biotin (Forward: 5'-TGGCCTGAGCTGTCAAAAC-3'; Reverse: 5'- GCGCGACTGGTTAGATCTTG-3'), and immobilized on Dynabeads Streptavidin (Dynal Invitrogen) according to the manufacturer's instructions. For each reaction, 40 µg nuclear extract was incubated with 1µg DNA probes in binding buffer [20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol and 2 µg poly(dI-dC) ] in a final volume of 400µl. The beads were extensively washed with binding buffer and then boiled in 25 µl

of 2x loading buffer before SDS-polyacrylamide gel electrophoresis followed by immunoblotting.

### 4.4.6. Chromatin immunoprecipitation assays (ChIP)

ChIP assays were carried out as previously described (Prince et al., 2004). Briefly, WI-38 cells (60%-70% confluence) were serum starved 24 hr prior to treatment with TPA or vehicle. Cells were fixed in 1% formaldehyde after 8 hr treatment. The chromatin was extracted, sonicated and immunoprecipitated using antibodies against Sp1. DNA precipitation was analyzed by PCR using human TBX2 specific primer pairs (Forward: 5'-TGGCCTGAGCTGTCAAAAC-3'; Reverse: 5'- GCGCGACTGGTTAGATCTTG-3').

# CHAPTER 5:

# CONCLUSION

The T-box family of transcription factors play a central role in embryonic development and is an important aspect of developmental biology. T-box members are expressed in specific cell types and are required for the development of their associated tissues and organs (Dobrovolskaïa-Zavadskaa, 1927). Their important regulatory roles in development have been demonstrated by mutational studies where mutant alleles, including heterozygotes, commonly give a phenotype. In humans for example, ulnar-mammary syndrome is caused by mutations in *TBX3* (Bamshad et al., 1997), Holt-Oram syndrome is linked to haplo-insufficiency of *TBX5* (Li et al., 1997) and mutations within *TBX1* have been associated with DiGeorge syndrome (Jerome et al., 2001; Lindsay et al., 2001; Mersher et al., 2001).

Human TBX2 has not yet been linked to any known genetic syndrome but has been implicated in limb, heart and mammary gland development (King et al., 2006; Plageman and Yutzey, 2005; Rowley et al., 2004). Targeted mutagenesis to investigate *Tbx2* function in mice have shown that heterozygous mutants appear normal while homozygous mutants die between 10.5 and 14.5 days post coitum of cardiac insufficiency (Plageman and Yutzey, 2005). TBX2 has also been implicated in tumourigenesis because its expression is deregulated in many melanoma, breast and pancreatic cancers (Mahlamaki et al., 2002; Packham and Brook, 2003; Sinclair et al., 2002; Vance et al., 2005) and it can suppress senescence through a mechanism involving its ability to repress expression of the cell cycle regulatory genes, $p19^{ARF}$ and $p21^{WAF1/CIP1/SD11}$ (Jacobs et al., 2000; Prince et al., 2004). Understanding the molecular role of Tbx2 in embryonic development and its impact on cell cycle control continues to represent a major challenge, in part, because its target genes and transcriptional regulation are still poorly characterised.

The present study thus contributes signifcantly to this knowledge base by firstly providing evidence that the *COL1A2* gene is a candidate target of Tbx2, and that Tbx2 may exert its effect on this gene as a co-repressor depending on the cell context and/or the species. Secondly, by cloning the 5'-regulatory region of the human TBX2 gene and

characterising the proximal promoter region of the gene, thus setting the stage for studying the regulation of the TBX2 gene. Finally, this study reveals that TBX2 gene expression is stimulated by the PKC signalling pathway through a mechanism involving chromatin remodeling via histone H3 phosphorylation.

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# APPENDIX 1

```
-1606   gtagggataacggtcgcagaggccatactgcttccgccggtcccgaatccgcactgccaggagagatccg

-1536   gccttcaaacgctgctcccaacgttcaacaacgaagtgccctgcgaccggtgtgcgcagtgggcgggcgc

-1456   ctgcatgggtcaatgttggatgtacgcgcccaccacgcggttgtacgggtgggaatgtcacccgtggcta

-1386   gtgcatgctctgtcttttccttcagaaCacgaccttcaggaatcagcctgagtgttcgcgcccgagcccg

-1316   attggaagcaggtgcgtggtcgcttcactctccccgtgcacaccttgagttatagctctcgctgcgcaca

-1246   gagggcaccacacggggcccgacacacacacacacacacacacacacacacacacaccatcacttcatgg

-1176   gggggaaactgaggctgccggaagacagaaaacgaagactgatcacagaagaggggcttcaatcacagg
                                    MluI
-1106   tgctcgcagacaccccaactccctcacgcgttactcagcactccttaacttaggagacgtgcgcttccga

-1036   gagatgagaagaaactaaaaaagtggttggcagggcgaagagaggaagccggtatttcggaagaaggaga
                      KpnI
 -966   ctttcgtctccggtaccggatagggcgaaaattcgttgggtcgcgggtggagaatccggggtccgcccgc

 -896   gcggtgccgccctgtgctcagcgaaaggtgcgctcaccttcctggccccgaggaCccgctcctcccggcc

 -826   gctggaaagctcagggcggggcccgggcctgcggagaactccaggttccttccgagtcttccggtgaagg

 -756   cgggcgcgggtagggggtccccgcgtttccccagcgcaaggccgcgtcccgctcccgcccgcgccggccc

 -686   cagcagggctcggccatggctccactcaccagctcccgccacctgggatccgctcccggacctcggccgc

 -616   ccgagcctcccgactcgcccgcccaccggcctcgctttccagtgcctgctgtctctttccggggcgcagg

 -546   gacccccaggcggcgccacgacccggagctgggtcggaggccaggctggggctcgggccagagcggccgg

 -476   gcctctgggcgcgggagaaggagggcgccccctctccgctcggggtcgtgtcaatgctttgcacttgggg
                                                SmaI
 -406   ccggcgtgcggctgcgggtccttccccaaggccccgggacccgggctcccctcgcctcaggccctttcgg

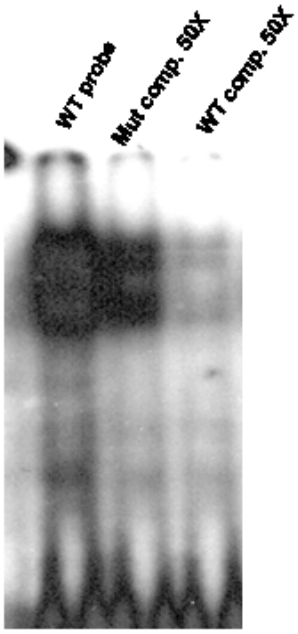
 -336   cgggtcaggtcggccctccgcgctctccagtc*cggcgcagg*cagccgcaggcgcgggcgggcggtggggg
                                                      SspI
 -266   ccggggcaggaggaggggtctcggggcccggcggcccgtcattggttaatattttattctgttgacatgt

 -196   tttcttactgctgaggcttccgacaccttctcccaggcccccctcccggccggagcttggcctgagctg

 -126   tcaaaaccccgcccccggagacccacaattggtccaaaaagcgtaaaatcagcaatcaaggggggcctgg
                                                                        +1
  -56    ctcgttagcgcaggggatccgagctgggcaggacatgtgagatagtcacagttttcCAGAGATCACGAC
        BglII
        AGATCTAACCAGTCGCGCG
```

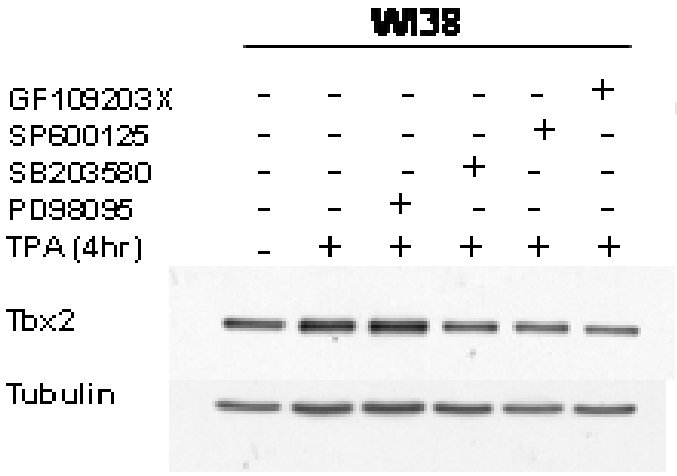
The DNA sequence of the 5'-flanking region of human TBX2 gene cloned from WI38 cell. Recognition sites for restriction enzymes BglII, SspI, SmaI, KpnI, and MluI are labeled. Nucleotides are numbered relative to the transcription initiation site (+1).

# APPENDIX 2




The nuclear factors of HT1080 cells binding to putative E-box element

# APPENDIX 3




TPA stimulated TBX2 gene expression in PKC-dependent manner in WI-38 cells at 4hr.

# APPENDIX 4

**Oligonucleotides used to generate mutant TBX2 proximal promoter by site-directed mutagenesis**

| Oligonucleotide | sequence (5' to 3') |
|---|---|
| Mut A Forward | CCGGCCGGAGCTTGGCCgttGCTtTCAAAACCCCGCCC |
| Mut B Forward | TGGCCTGAGCTGTCAAAgtaCCGCCCCCGGAGACCCAC |
| Mut C Forward | GACATGTTTTCTTACTGCTGAttaTTCCGACACCTTCTCCCA |
| Mut D Forward | GACATGTTTTCTTACTGCcaAGGCTTCCGACACCTTCTC |
| Mut E Forward | GTTTTCTTACTGCTGAGGCTctCGACACCTTCTCCCAG |
| Mut F Forward | CTGTTGACATGTTTTCTTACacCTGAGGCTTCCGACACCT |
| Mut G Forward | CCAAAAAGCGTAAAATCAGCAAgttAGGGGGGCCTGGCTCGT |
| Mut H Forward | TGGTCCAAAAAGCGTAAAATtttCAATCAAGGGGGGCCTGGCTC |
| Mut I Forward | CAGCAATCAAGGGGGaCgTGGCTCGTTAGCGCA |
| Mut J Forward | CAGCAATCAAGGGGGGCCatGtTCGTTAGCGCAGGGGA |
| Mut K Forward | GTCAAAACCCCGCCCCCGtttACCCACAATTGGTCCAA |
| Mut L Forward | AGGGGGGCCTGGCTCGTTAtttAGGGGATCCGAGCTGGGCA |

The mutations are indicated with lowercase letters

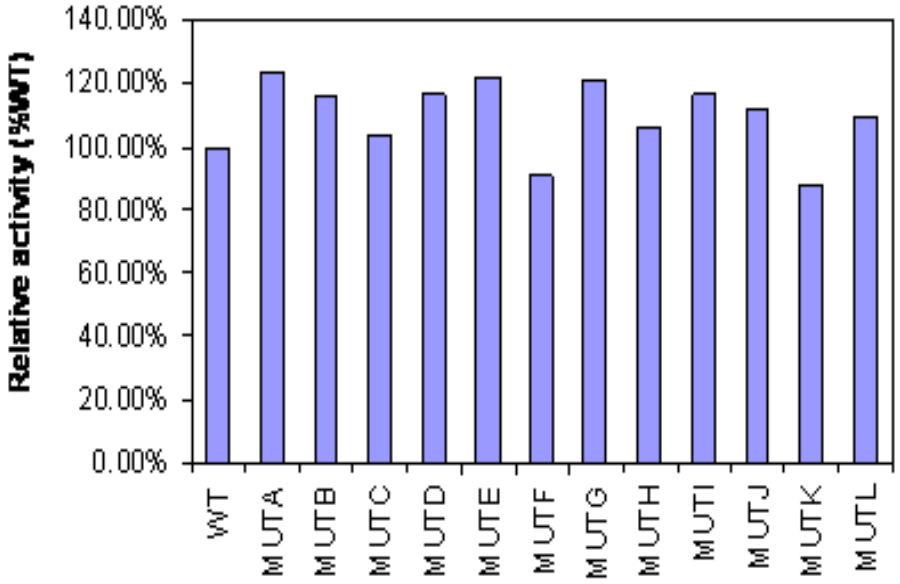
```
 -216
ATTTTATTCTGTTGACATGTTTTCTTACTGCTGAGGCTTCCGACACCTTCTCCCA Human
ATTTTATTCTGTTGACATGTTTTCTTACTGCTGAGGCTTCCGACACCTTCTCCCT Mouse
 -208                          MutF MutD MutC       MutE     *

GGCCCCCCCTCCCGGCCGGAGCTTGGCCTGAGCTGTCAAAACCCCGCCCCCGGAG Human
GGCCTCCCGTCCCGGCCGGAGCTTGGCCTGAGCTGTCAAAACCCCGCCCCCGGAG Mouse
    *   *                        MutA      MutB          MutK

ACCCACAATTGGTCCAAAAAGCGTAAAATCAGCAATCAAGGGGGGCCTGGCTCGT Human
ACCCACAATTGGTCCAAAAAGCGTAAAATCAGCAATCAAGGGGGGCCTGGCTCGT Mouse
                               MutH  MutG      MutI  MutJ+1

TAGCGCAGGGGATCCGAGCTGGGCAGGACATGTGAGATAGTCACAGTTTTCCAGA Human
TAGCGCAGGGGATCCGAACAGGGCAGGACATGTGAGATAGTCAGAGTTTTCCAGA Mouse
  MutL            * *                          +1

                              +32
GATCACGACAAGATCTAACCAGTCGCGC                            Human
GATGACGACAAGATCTAACCAGTCGCGC                            Mouse
   *                          +40
```




500 ng of the proximal TBX2 promoter (-216/+32) construct, its mutant construct were co-transfected with 50 ng pRL-TK into HT1080 cells. Firefly luciferase activity was normalized to renilla luciferase activity. The basal activity of the -216/+32 construct was set at 100%..