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Review

T-box transcription factors in cancer biology

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ABSTRACT

The evolutionarily conserved T-box family of transcription factors have critical and well-established roles in embryonic development. More recently, T-box factors have also gained increasing prominence in the field of cancer biology where a wide range of cancers exhibit deregulated expression of T-box factors that possess tumour suppressor and/or tumour promoter functions. Of these the best characterised is TBX2, whose expression is upregulated in cancers including breast, pancreatic, ovarian, liver, endometrial adenocarcinoma, glioblastomas, gastric, uterine cervical and melanoma. Understanding the role and regulation of TBX2, as well as other T-box factors, in contributing directly to tumour progression, and especially in suppression of senescence and control of invasiveness suggests that targeting TBX2 expression or function alone or in combination with currently available chemotherapeutic agents may represent a therapeutic strategy for cancer.

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Contents

1. Introduction

The T-box gene family is one of the most prominent gene families in the field of embryonic development and not surprisingly its members are highly conserved in evolution ranging from hydra to human [1,2]. Based on phylogenetic and expression studies, the family has been divided into five subfamilies, namely *T*, *Tbx1*, *Tbx2*, *Tbx6* and *T-brain1* [3]. All T-box transcription factors are characterised by a highly conserved DNA binding domain, called the T-box, which binds to the core sequence GGTGTGA, referred to as the T-element [2]. T-box factors have been shown to function as either transcriptional activators or repressors although there are some that appear capable of both.

Genetic studies in flies, worms, fish, mice, dogs, and humans have shown that T-box proteins are generally expressed in certain cell types of specific organs and are also necessary for the development of these structures [3,4]. To date, 18 T-box transcription factors have been identified in mammals and several members have been implicated

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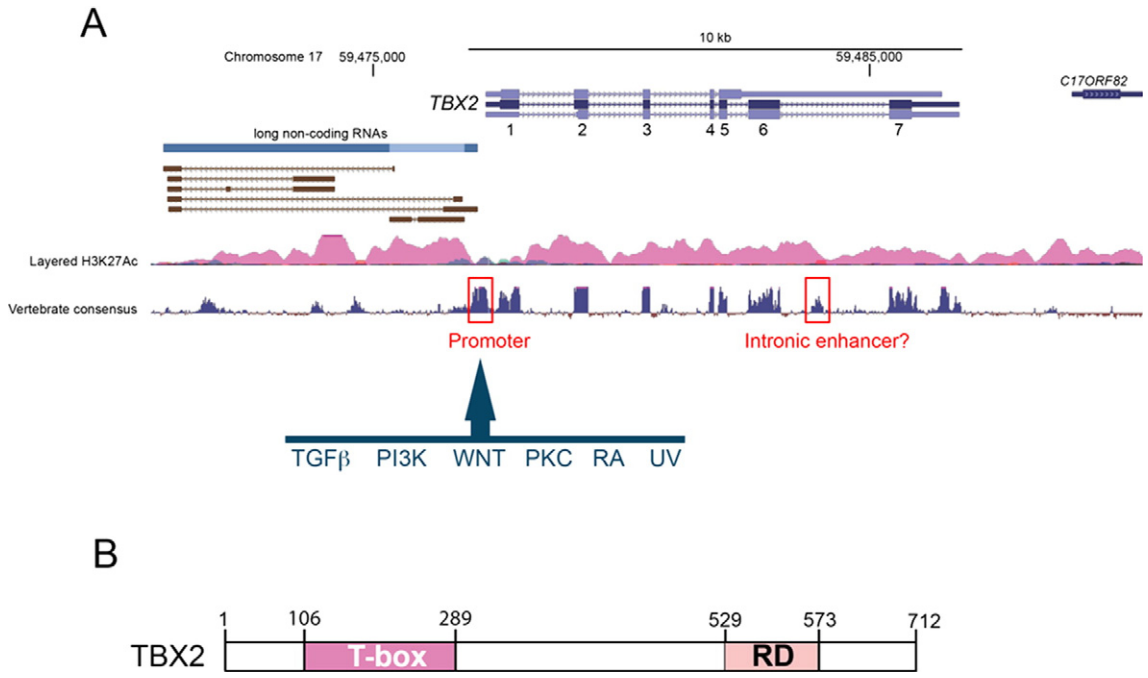

Fig. 1. TBX2 gene and protein. Schematic representation of the (A) *TBX2* gene on human chromosome 17 including exons (filled boxes) and introns (lines). The presence of an uncharacterised long-non-coding mRNA located 5′ to the *TBX2* gene is also indicated, together with the location of the epigenetic H3K27 histone mark, a hallmark of active gene expression, and evolutionarily conserved non-coding regions (promoter and putative enhancer) likely to play a key regulatory role in *TBX2* expression. The genomic data was taken from the UCSC genome browser, and signals that regulate *TBX2* expression are indicated with the arrow pointing to the *TBX2* promoter; and (B) TBX2 protein showing the position of the T-box and the dominant repression domain (RD). Numbers indicate the amino acids numbers at the boundaries of each domain.

in early foetal development, ranging from the specification of the primary germ layers to organogenesis [3]. The important role of T-box factors in embryonic development is well documented and has been extensively reviewed [3,5–8]. Several T-box factors are directly linked to human developmental disorders which emphasises their critical role in embryonic development and the phenotypes associated with these reveal the vast array of developmental processes that they regulate. For example, mutations leading to haploinsufficiency of *TBX1*, *TBX3*, *TBX5* and *TBX6* result in DiGeorge, ulnar–mammary, Holt–Oram and dominant spondylocostal dysostosis syndromes respectively, and mutations in *TBX19* and *TBX22* are responsible for adrenal insufficiency and X-linked cleft palate with ankyloglossia respectively [9,10].

2. T-box factors in cancer

Phenotypic plasticity, invasiveness, and proliferative capacity are key features of cancer biology and are reminiscent of the cellular processes that occur during embryonic development. It is therefore not surprising that important developmental regulators, including members of the T-box gene family, have been implicated in carcinogenesis [11,12]. A growing list of studies have reported deregulated levels of T-box factors in different cancer types and inappropriate T-box factor expression has been directly linked to oncogenesis. Indeed, several T-box factors, including *Brachyury*, *Eomes*, *TBX1*, *TBX2*, *TBX3*, *TBX4*, *TBX5* and *T-bet* (*TBX21*) [13–21], possess tumour promoting or tumour suppressor functions. Of these the best-characterised T-box factor implicated in cancer progression is TBX2.

2.1. TBX2

2.1.1. Gene and protein structure of TBX2

TBX2 is a member of the subfamily which includes *TBX3*, *TBX4* and *TBX5* and is evolutionarily most closely related to *TBX3* [22]. *TBX2* maps to chromosome *17q23* in humans and chromosome *11* in mouse [23]. The human *TBX2* gene spans 9.53 kb and is processed into a number of transcripts (Fig. 1A), the longest comprising 7 exons that give rise to a 3339 bp long transcript that encodes a 712 amino acid protein (Fig. 1B). The human and mouse TBX2 proteins are 96% homologous which further highlights the previously mentioned evolutionary conservation among T-box factors [2]. Intriguingly, the human gene is bordered immediately upstream from its promoter by a number of divergently transcribed long non-coding RNAs that are widely expressed in different tissues. Given the proximity of the non-coding RNA transcription initiation sites to the highly evolutionarily conserved TBX2 promoter, it is possible that they may share regulatory elements, though their function is currently unknown. In addition, based on sequence conservation between species, there is a potential uncharacterised intronic enhancer in intron 6.

Early investigation of TBX2 as a transcription factor indicated that it can act as a transcriptional repressor [24], a property it shares with TBX3, but not with the related sub-family members TBX4 and TBX5 that activate transcription. Thus in addition to its DNA-binding domain, the T-box that is shared with other family members, Tbx2 contains two regions that confer transcription repression, one located within the N-terminal 52 residues and the second in the C-terminal region (Fig. 1B) [25]. A domain within the T-box was able to weakly activate a synthetic promoter [25] and a recent study reported that Tbx2 is able to transcriptionally activate the transforming growth factor β2 (Tgfb2) promoter [26]. Consistent with this, TGFB2 is up-regulated in a microarray study when TBX2 is overexpressed [27]. Although these genes may represent targets, the genome-wide repertoire of TBX2 binding sites remains to be determined. Consequently it remains an open question whether some genes may be repressed while others activated by TBX2, especially when considering the complexities of T-box factor binding to regulatory elements within a chromosomal context where the relative positions of multiple regulatory motifs controlling specific genes combined with the local epigenetic landscape may affect the outcome in terms of activation or repression.

Tbx2 plays a central role in the development of the fore- and hindlimbs, mammary glands, lung, heart, eye and kidney [3,23]. *TBX2*'s importance in development is underscored by observations that *Tbx2*

homozygous mutant mouse embryos are not viable and die in utero of heart abnormalities though heterozygous mutant mice survive [28,29]. *TBX2* is also expressed in healthy adult tissues such as the kidney, lung, prostate, spleen and ovaries [30,31] but there are no reports as to what genes are regulated by Tbx2 in these tissues. Two recent studies have described microdeletions on chromosome *17q23*, which affect *TBX2* and *TBX4*, to be associated with an as yet unnamed new syndrome characterised by microcephaly, postnatal growth retardation, hand, foot, and limb abnormalities and heart defects [32,33].

2.1.2. TBX2 in cancer

Overexpression of TBX2 in cancer was first demonstrated in a range of breast cancer tissues and cell lines [34–37] and high TBX2 expression correlates with a poor prognosis [38]. Subsequently TBX2 overexpression has been observed in pancreatic cancer, colorectal, melanoma, endometrial, ovarian and cervical cancers and most recently in rhabdomyosarcomas. Its overexpression in pancreatic cancer was shown in cancer cell lines [39] and in tumour sections and was positively correlated with tumour stage, differentiation [40] and metastasis [41]. Recently, TBX2 was suggested as a prognostic marker for colorectal cancer as its overexpression in colorectal tumour tissue strongly correlated with tumour stage, invasion, distant metastasis and a very poor prognosis [42]. TBX2 overexpression also occurs in a subset of melanomas [43–45] and a high-throughput microarray study revealed that on average TBX2 is expressed significantly higher in melanomas than in other tumour samples tested [46]. While the overexpression of *TBX2* in endometrial and ovarian cancer correlates with tumour stage, little is known about *TBX2*'s role and regulation in these cancers [47,48]. TBX2 is also overexpressed in 52% of cervical squamous cell carcinoma tissue sections and is especially high in sections of Human Papillomavirus (HPV)-16 positive tumours. Consistent with reports for pancreatic and colorectal cancer, elevated TBX2 levels in cervical cancer were associated with increased metastasis [49]. Interestingly, Schneider et al. [50] showed that TBX2 might be involved in the regulation of the life cycle of HPV a key causative agent in cervical cancer. They identified TBX2 as a co-factor of the HPV capsid protein L2, with TBX2 directly binding and repressing the long coding region (LCR) to regulate expression of the HPV early genes E6 and E7, repression that was enhanced when TBX2 was co-expressed with L2. Whether the interaction between TBX2 and L2 in regulating the viral E6 and E7 genes plays an important role in establishing or maintaining HPV infection in vivo is unclear, but it is possible that it contributes to the oncogenic properties of HPV infection.

2.1.2.1. TBX2 in bypassing senescence and promoting proliferation. Numerous studies have demonstrated that TBX2 functions as a potent growth-promoting factor, in part due to its ability to bypass senescence and to repress key negative regulators of the cell cycle (Fig. 2). Jacobs et al. [35] were the first to suggest a direct role for TBX2 in cancer when they showed that Tbx2 can bypass the onset of senescence in MEFs. Senescence is broadly defined as the physiological programme of irreversible growth arrest and is an important barrier to malignancy as it prevents cells from becoming immortal [51–55]. Senescence can be triggered by the shortening of telomeres as a consequence of cell division (replicative senescence), or by cellular stresses (accelerated senescence/oncogene induced senescence). Although the molecular pathway(s) leading to senescence differ in species and cell types, there is general consensus that the $p14^{ARF}$–p53–p21 and $p16^{INK4A}$–RB signalling cascades are key to the process [56,57]. While $p14^{ARF}$ up-regulates p53 protein levels by inhibiting the ubiquitin ligase MDM2, $p16^{INK4A}$ inhibits CDK4 that drives cell cycle progression by phosphorylating the retinoblastoma protein (RB1) leading to activation of E2F transcription factors and consequently expression of S-phase genes. Tbx2 can bypass senescence through its ability to repress cyclin-dependent kinase inhibitor 2A (Cdkn2a) that encodes $p19^{ARF}$ ($p14^{ARF}$ in humans) (Fig. 2) [35]. However, as many cancers lack functional $p16^{INK4A}$ and $p14^{ARF}$ coding sequences, a key question arose as to what role TBX2 would have in senescence bypass in human cancers. This issue was resolved when Prince et al. [58] demonstrated that Tbx2 can also bypass senescence through directly repressing p21 by binding to its transcription initiation site (Fig. 2). Subsequent studies in MEFs confirmed that Tbx2 can bypass senescence through repressing $p19^{ARF}$ [59] and p21 [44,60]. A key role for TBX2 in senescence bypass was reinforced when it was revealed that TBX2 is a PML-repressible E2F-target gene in WI38 primary human diploid fibroblasts [61]. In these cells, repression of TBX2 via a PML recruitment to its promoter by a p130/E2F4 repressor complex led to senescence, but direct PML–TBX2 interaction also antagonized PML-driven senescence, highlighting the presence of an autoregulatory loop.

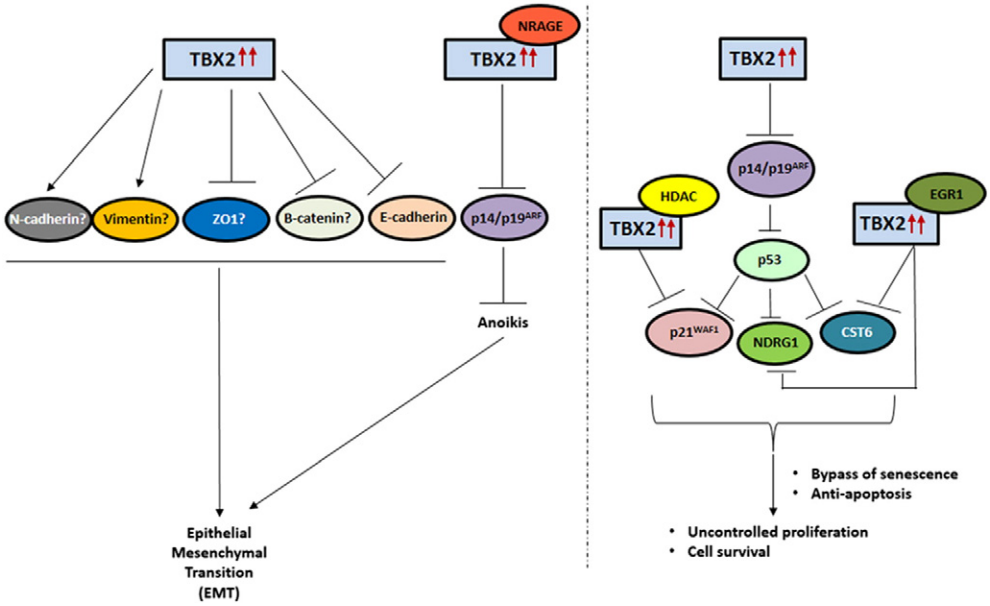

Fig. 2. TBX2 oncogenic roles mediated through its known co-factors and target genes.

Melanomas are neoplasms derived from melanocytes and the bypass of senescence associated with genetic lesions is a critical step in melanoma formation. For example, *CDKN2A*, which encodes the tumour suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$, is mutated in approximately 40% of familial melanoma [62,63] and *CDK4* is the second most common susceptibility gene in melanoma [64–66]. The overexpression of TBX2 in a subset of melanomas raised the question as to whether it could contribute to melanomagenesis by bypassing senescence. This was a particularly attractive possibility because it was previously shown to inhibit senescence in mouse cells but whether this was possible in human melanoma cells overexpressing TBX2 was initially unclear. Indeed, two independent studies have shown that TBX2 functions as an anti-senescence factor in mouse and human melanomas [44,67]. Silencing TBX2 in a vertical growth phase melanoma cell line induced several features of senescence, including heterochromatic foci and β-galactosidase activity, and knocking it down in metastatic melanoma cell lines resulted in a strong reduction of proliferation of adherent and non-adherent cells [67]. Vance et al. [44] demonstrated that B16 mouse melanoma cells in which Tbx2 was depleted senesced via a mechanism in which Tbx2 represses *p21* gene expression through its ability to recruit histone deacetylase 1 (HDAC1) to the initiator of the *p21* promoter (Fig. 2). It is also worth noting that a different study by Vance et al. [68] using the same mouse melanoma cells showed that hypo-phosphorylated (active) Rb1 binds Tbx2 and enhances its repression of *p21*. These findings convincingly show that TBX2 plays a causative role as a pro-proliferative factor in melanomagenesis and identify TBX2 as a promising anti-cancer drug target.

Endogenous *TBX2* also acts as an important pro-proliferative factor in breast cancer cell lines derived from different subtypes of breast tumours. Tumours of the breast are in part grouped based on expression levels of receptors for oestrogen, progesterone or human epidermal growth factor 2 (HER2) and breast tumours which lack expression of all three receptors are classified as triple negative [69,70]. Silencing TBX2 in oestrogen receptor-positive MCF-7 cells reduced substrate-dependent and -independent cell proliferation [67]. These results were confirmed in two additional breast cancer cell lines representing HER2 receptor positive (MDA-MB-453) and oestrogen and HER2 receptor positive (BT474) tumour subtypes [71]. This suggests that the pro-proliferative role of TBX2 is independent of oestrogen and HER2 signalling which correlates with available microarray data from a range of breast cancer tissues which show that TBX2 overexpression is independent of hormone receptor status [38]. The molecular mechanism underpinning the pro-proliferative role of TBX2 in breast cancer was shown to result from it repressing the tumour suppressor *N-myc downregulated gene 1* (*NDRG1*) and *cystatin 6* (*CST6*) in co-operation with early growth regulator 1 (EGR1) (Fig. 2) [71,72]. Furthermore, the authors showed that when *TBX2* or *EGR1* were silenced, apoptotic and senescence markers increased and this effect could be mimicked by overexpressing *NDRG1* in *TBX2*-expressing cells [71]. CST6 has a strong affinity for a well-reported cancer-linked enzyme called legumain which has been shown to be associated with poor prognosis in multiple cancer types. This provides a novel potential therapeutic opportunity to target an enzymatic activity controlled downstream of TBX2.

The recent discovery that TBX2 is highly upregulated in rhabdomyosarcomas is particularly interesting in light of the increasing evidence that T-box factors play a pivotal role in embryonic development and cancer. Rhabdomyosarcomas are soft tissue sarcomas, which have common features with developing skeletal muscle cells. In this cancer TBX2 recruits histone deacetylase 1 (HDAC1) to the promoters of the muscle specific genes *MyoD* and *Myogenin* that encode key transcription factors that affect the myogenic programme in normal myoblasts and *p21* and $p14^{ARF}$ in rhabdomyosarcoma cells [73]. Consistent with these observations, overexpression of TBX2 in normal myoblasts strongly inhibited myogenesis whereas depletion of TBX2 in the rhabdomyosarcoma cells decreased cell proliferation in vitro. Especially striking was the observation that depletion of TBX2 also prevented tumour growth in vivo.

The ability of TBX2/Tbx2 to repress negative regulators of the cell cycle suggests that it contributes to oncogenesis through deregulating the cell cycle. Indeed, Tbx2 is tightly regulated during the cell cycle with its levels increasing during late S-phase, peaking at G2 and dropping in mitosis [74], and ectopic expression of Tbx2 caused mitotic defects in three different cell lines [75,76]. Importantly, Davis et al. [75] went on to show that TBX2-overexpressing cells bypass the tetraploidy checkpoint leading to genetic instability and polyploidy which are key hallmarks of cancer [77].

2.1.2.2. TBX2 in the DNA damage pathway. Up to 10% of all breast cancer cases can be explained by the inheritance of mutations in one or more breast cancer susceptibility gene [78–80]. Importantly, most of these genes are involved in the execution of error-free DNA repair suggesting that the impairment of this process is critical in the development of breast cancer [81–84]. Known susceptibility variants include high-penetrance mutations leading to the loss of function in the tumour suppressor activity of *BRCA1* and *BRCA2*, which account for 52% and 32% of all familial breast cancers, respectively [79,85]. In addition, susceptibility variances in *CHK2*, *PALB2*, *ATM* and *BRIP1* are regarded as medium penetrance mutations and can together explain 5% of hereditary breast cancer cases [79]. *CHK2* encodes a kinase that phosphorylates p53 and BRCA1 and through BRCA1 regulates the repair of DNA double-strand breaks [84,86]. *PALB2* encodes a protein that facilitates BRCA2-mediated DNA repair by promoting localisation and stability of BRCA2 [87]. The ATM protein kinase initiates the cell cycle arrest which allows for DNA repair and the activation of the DNA repair process itself and this is mediated through the phosphorylation of multiple proteins including p53, CHK2, BRCA1 and BRCA2 [88,89]. *BRIP1* encodes an enzyme that interacts with BRCA1 and also plays a role in checkpoint control [90]. The loss of function of any one of these genes is linked to genomic instability resulting in an increased risk in developing breast cancer [81–84]. Interestingly, *TBX2* is preferentially amplified in *BRCA1* and *BRCA2*-associated breast cancers [36] and has been linked to the DNA damage response [23]. In MCF-7 breast cancer cells silencing TBX2 abrogates the cisplatin-induced ATM–CHK2–p53 signalling pathway which prevents an S-phase arrest and DNA repair [91]. Importantly this leads to cells entering mitosis with damaged DNA and consequently undergoing mitotic catastrophe and cell death. These data suggest that TBX2 may promote oncogenesis in part through disabling the DNA damage response and through this promoting inappropriate survival of cells with damaged DNA. As such it might also be expected that factors that regulate TBX2 expression would also confer differential sensitivity to DNA damaging agents. For example, the master-regulator of the melanocyte lineage, the microphthalmia-associated transcription factor MITF regulates TBX2 expression [24], and silencing MITF sensitises melanoma cells to the chemotherapeutic drugs cisplatin and docetaxel [92]. While the mechanism by which MITF contributes to resistance to DNA damaging chemotherapeutics and microtubule poisons in melanoma cells may be related to its role in the DNA damage pathway and DNA replication [93,94], it is also possible that altered expression of TBX2 downstream of MITF contributes to cisplatin resistance [75,91].

Consistent with a potential role for Tbx2 in the DNA-damage response, TBX2 protein levels are upregulated by the DNA damage stress pathway [23,95]. UVC-induced DNA damage in MCF-7 breast cancer cells leads to an increase in TBX2 mRNA and protein levels arising in part because of p38-mediated phosphorylation of TBX2 on serine residues 336, 623 and 675. Phosphorylation also resulted in the TBX2 protein moving from the cytoplasm to the nucleus and enhanced the ability of TBX2 to repress its target genes, *p21* and $p14^{ARF}$.

2.1.2.3. TBX2 in EMT. During metastasis tumour cells acquire traits of so called epithelial to mesenchyme transition (EMT) [96] and a recent study by Wang et al. [38] implicated TBX2 as an inducer of EMT in breast cancer. Ectopic expression of *TBX2* in normal breast epithelial cells

resulted in a decrease in the epithelial markers, E-cadherin, β-catenin and zonula occludens 1 (ZO1), and an induction of the mesenchymal proteins, N-cadherin and vimentin (Fig. 2). In vitro, the cells ectopically expressing TBX2 had an increased ability to invade and migrate. Consistent with this data, silencing TBX2 in metastatic breast cancer cells had the opposite effect by reducing expression of mesenchymal markers and the invasion and migratory ability of the cells in vitro. Importantly, when these TBX2 knockdown cells were injected into the tail vein of nude mice, no lung metastasis could be detected as compared to control cells that formed pulmonary tumours. Based on chromatin immunoprecipitation (ChIP) analysis and luciferase assays Wang et al. [38] proposed that TBX2 contributes to EMT by directly binding and repressing the promoter of *CDH1* (*E-cadherin*). This is supported by previous work in melanoma cells showing that ectopic TBX2 could directly repress E-cadherin expression, though in melanoma, E-cadherin was primarily suppressed by TBX3 [45]. Anoikis resistance plays a key role in EMT, because it allows for cell detachment without triggering apoptosis. Importantly, TBX2 may also promote EMT of breast cancer cells by co-operating with the anoikis suppressor NRAGE; a complex of NRAGE and TBX2 binds and represses the $p14^{ARF}$ promoter through a T-element in EMT-like cells and the re-expression of $p14^{ARF}$ sensitised these cells to anoikis (Fig. 2) [97].

2.1.2.4. Signalling pathways that up-regulate TBX2 expression in cancer. While the evidence that TBX2 overexpression is critical to the cancer process is accumulating, there is little information on the mechanisms responsible for its overexpression. *TBX2* maps to chromosome *17q23*, a region that is amplified in a subset of melanomas [98], breast [99], ovarian [47], and pancreatic [100] cancers and genetic amplification of *TBX2* leads to the overexpression of TBX2 in the MCF-7 breast cancer cell line as well as a number of primary breast tumours and in 50% of pancreatic cancer cell lines tested [34–37,39]. While genetic amplification may partly account for TBX2 overexpression in some cancers, there is evidence that TBX2 levels may also be increased due to the deregulation of signalling pathways controlling its expression (Fig. 1A).

Ismail and Bateman proposed in 2009 that TBX2 may be regulated by the PI3K pathway which is known to promote many cellular processes contributing to cancer formation [27]. Classically, the activation of a growth factor receptor on the cell surface activates PI3K, which phosphorylates inositol phospholipids to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the inner side of the cell membrane. PIP_3 interacts with the pleckstrin homology (PH) domains of several signalling molecules and recruits these to the inner side of the cell membrane. The serine/threonine kinase AKT (also known as protein kinase B) is one of the recruited signalling molecules and is activated through phosphorylation by the protein serine/threonine kinases 3′-phosphoinositide-dependent kinase (PDK) 1 and 2. The study by Ismail and Bateman [27] demonstrated that stable transfection of an adrenal carcinoma cell line (SW13) with the growth factors *FGF4* or *progranulin* resulted in the activation of the PI3K–AKT pathway and in increased TBX2 mRNA and protein levels. Importantly, the same study showed that TBX2 overexpression promoted anchorage-independent growth in SW13 cells. Consistent with these findings, when the authors treated the FGF4 overexpressing cells with a PI3K inhibitor, *TBX2* mRNA levels decreased. While this study showed that *TBX2* is regulated by the PI3K pathway it did not provide any mechanistic detail as to which transcription factor(s) was involved in coupling PI3K signalling to the *TBX2* promoter. However, since PI3K signalling plays a crucial role in cancer progression, cooperating with pro-proliferative signals by driving senescence bypass [101], the possibility that TBX2 may play a key role in implementing the anti-senescence signal generated by PI3K signalling is very attractive.

Signalling pathways regulated by WNTs play significant roles in cellular processes during embryonic development and tumourigenesis [102,103]. Binding of WNT molecules to their receptors on the cellsurface regulates signalling pathways through inhibiting the degradation of the transcriptional regulator β-catenin. There are indications that the WNT/β-catenin pathway activates *TBX2* expression during embryonic development and in cancer. For example, in response to Wnt/β-catenin signalling *Tbx2* is activated by the bone morphogenetic protein (Bmp) 2 and bone morphogenetic protein 4 during heart patterning [104]. Importantly, the inhibition of β-catenin degradation by lithium chloride in a pancreatic cancer cell line induced TBX2 mRNA and protein levels [40]. Senescence bypass mediated by WNT signalling may also be important in melanoma, where constitutive activation of the pathway prevents senescence of melanocytes in vitro and in vivo [105] though the relative contribution of WNT/ β-catenin-driven activation of TBX2 in these cells is unclear.

The transforming growth factor β1 (TGF-β1) pathway plays critical roles in a wide range of cellular processes including cell proliferation, apoptosis, differentiation and the immune response. This multifunctional cytokine generally exerts its effects by binding and activating the TGFβ receptors I and II, which initiate a cascade of signalling events frequently involving Smad proteins and co-factors but which can also be Smad-independent [106]. One of the well-known roles of TGF-β1 is its ability to inhibit the proliferation of epithelial cells but promote their migration [107]. TGF-β1 inhibits proliferation primarily by inducing a G1 cell cycle arrest through its ability to transcriptionally up-regulate expression of the cyclin dependent kinase inhibitors $p15^{Ink4b}$ and $p21^{Cip1}$ [108]. Its pro-migratory effects, however, appear to be mainly owing to its ability to regulate key players of EMT [109]. These anti-proliferative and pro-migratory roles of TGF-β1 are important in embryonic development, for example during branching morphogenesis of the mammary gland, as well as during oncogenesis [110,111]. A recent study by Wang et al. [38] in primary human mammary epithelial cells demonstrated that TBX2 mRNA and protein levels were increased following TGFβ treatment and the authors hypothesised and subsequently showed that TBX2 is a driver of EMT in breast cancer. Contrary to this finding which suggests a positive relationship between TGFβ and TBX2, the anti-proliferative effect of TGFβ on B16 melanoma cells was rescued by *Tbx2* overexpression [112]. However, while these results imply that TGFβ represses Tbx2 the authors did not confirm this by analysing Tbx2 mRNA or protein expression levels. Furthermore, while neither of the studies implicating TBX2 downstream of the TGFβ pathway provided a mechanism for this regulation they do suggest that the nature of this regulation may be species and/or cell type dependent.

TBX2 is also up-regulated by the PKC signalling pathway [113]. Enzymes belonging to the PKC family are lipid-dependent serine/threonine kinases that play key roles in cellular processes such as proliferation, cell cycle progression and apoptosis [114]. The PKC family is linked to cancer formation and progression, because many PKC activators can act as tumour promoters, which is supported by functional studies [115]. In the study by Teng et al. [113], the PKC pathway was stimulated in normal human fibroblasts by the tumour promoting agent TPA. The results suggest that in response to TPA treatment, PKC activates mitogen- and stress-activated kinase 1 (MSK1) which in turn phosphorylates histone H3 leading to chromatin remodelling and recruitment of Sp1 to the *TBX2* promoter and subsequent activation of TBX2 mRNA expression.

Retinoic acid (RA) plays a critical role during embryonic development and Sakabe at al. [26] recently reported that during murine outflow tract development ectopic RA expression represses Tbx2 transcription via direct binding to a highly conserved RARE located at +210 bp [26]. Interestingly, gene array analysis of B16 mouse melanoma cells treated with and without RA revealed that Tbx2 is activated by RA and that it is an early RA response gene [116]. This result was subsequently validated by qRT-PCR and luciferase reporter assays [117]. The apparent opposing responses of Tbx2 to RA could be explained by melanoma cells having a defective RA pathway

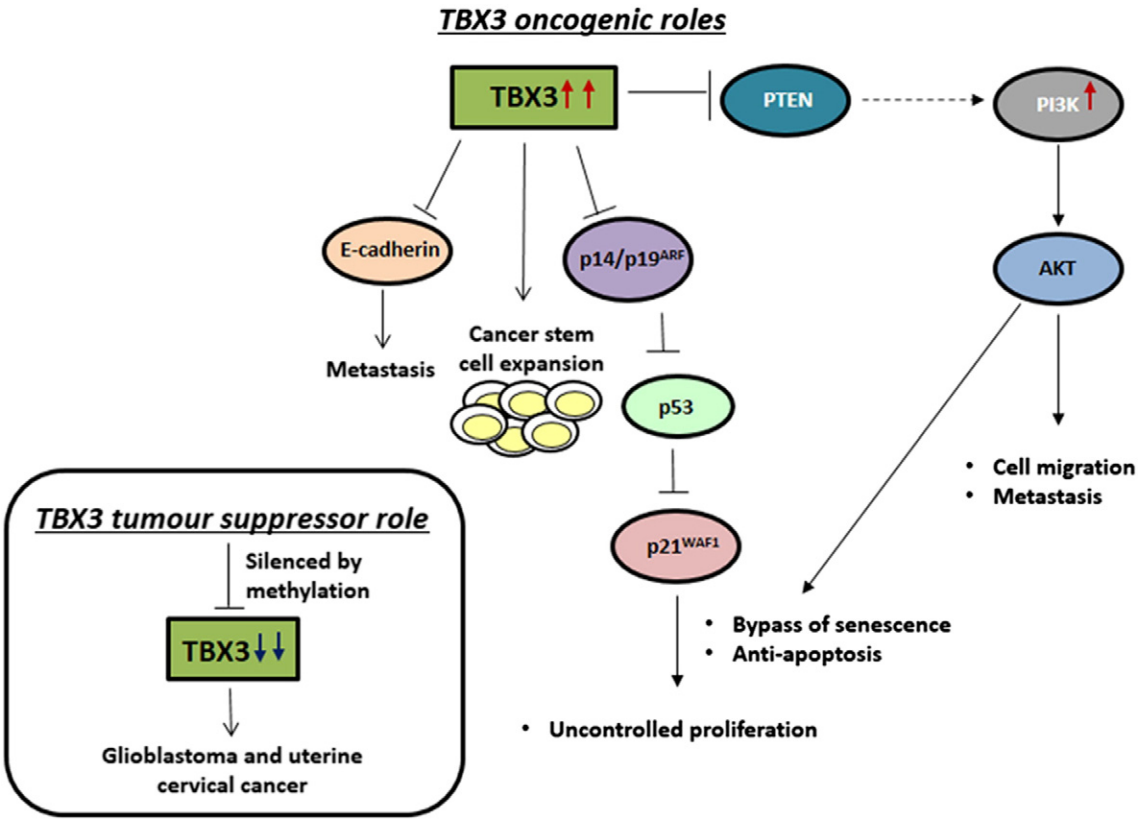

Fig. 3. Mechanisms by which TBX3 may promote or suppress tumour formation. Solid arrows represent established mechanisms and broken arrow represents proposed model.

which is highly likely because RA signalling is often compromised in tumourigenesis [118].

2.2. TBX3

Given the major role played by TBX2 in cancer progression, it is not surprising that its most-closely related family member TBX3 is also heavily implicated in this disease. Human *TBX3* is expressed in a number of tissues and organs including the limbs, jaw mesenchyme, salivary gland, adult adrenal gland, thyroid, foetal heart, lungs, mammary gland, liver, bladder, uterus, genitals, spleen, and nervous system [119]. Much information on the roles of TBX3 in development has been derived from the autosomal dominant human developmental disorder ulnar–mammary syndrome (UMS) and mouse models where Tbx3 has been mutated. UMS is characterised by malformations in axillary hair, abnormal dentition, mammary gland aplasia, loss of areola, limb abnormalities and defects in the heart, jaw and genitalia [9]. Consistent with the wide range of tissues to which it contributes during development, TBX3 overexpression in a number of adult tissues has been implicated in cancers including breast, melanoma, pancreatic, lung, ovarian, liver and head and neck cancers [45,120–128]. In addition, an extensive body of research has shown that TBX3 overexpression contributes to several aspects of the oncogenic process, in part, and like TBX2, through its ability to repress $p19^{ARF}$ ($p14^{ARF}$ in humans) [129], p21 [130], phosphatase and tensin homolog (PTEN) [122] and E-cadherin [45].

Early studies demonstrated that the stable expression of Tbx3 led to bypass of senescence and consequently to the immortalisation of mouse embryonic fibroblasts (MEFs) through the ability of Tbx3 to repress $p19^{ARF}$ (Fig. 3) [131,132]. Furthermore, the repression of $p19^{ARF}$ by Tbx3, which also correlated with decreased expression of p53 and p21, overrides apoptosis induced by the oncogene Myc in MEFs (Fig. 3) [132]. Moreover, silencing Tbx3 in rat bladder cancer cells reduced cell proliferation and induced apoptosis [133]. Similarly, Renard et al. [128] established that TBX3 mediates the pro-proliferative activity of the β-catenin signalling pathway in liver and colon cancer cells where the Wnt/β-catenin pathway is constitutively activated and drives tumourigenesis. TBX3's expression was also found to correlate with rapid cell proliferation, invasiveness and tumour size in uterine and cervical cancer [134]. Recent studies have also suggested that TBX3 may promote cancer cell migration at the expense of their proliferation i.e. that TBX3 may play an important role as a reciprocal switch between substrate dependent cell proliferation and tumour invasion [67,135]. However, the overexpression or knock down of TBX3 in mammary epithelial cells (MECs) apparently has little effect on apoptosis [136]. It is possible that the impact of TBX3 on proliferation and apoptosis may be cell context specific.

Importantly, Burgucu et al. [122] demonstrated that TBX3 directly represses the tumour suppressor PTEN that is an important negative regulator of the phosphatidylinositide 3-kinase (PI3K) pathway and through this indirectly regulates cell proliferation and survival (Fig. 3). As highlighted above, the PI3K pathway plays a major role in cancer by facilitating senescence bypass as well as enhancing cancer cell migration and metastasis [137,138]. TBX3-mediated repression of PTEN leading to elevated PI3K signalling may therefore make a major contribution to its capacity to facilitate senescence bypass and promote cell migration and invasion (Fig. 3).

TBX3 promotes melanoma migration and invasion through an ability to repress E-cadherin (Fig. 3) [45,67,135], with TBX3 mRNA expression increasing as cells progress from radial to the invasive vertical growth phase [45]. Consistent with this, overexpressing TBX3 in non-tumourigenic early stage melanoma cells was sufficient to promote tumour formation and invasion in vitro and in vivo and strongly correlated with decreased levels of E-cadherin [135]. Importantly, TBX3 lies downstream from BRAF, a serine/threonine kinase in the ERK/MAPK signalling pathway which is constitutively activated by mutation in 50% of melanomas with the common mutation being $BRAF^{V600E}$ [139]. While very little is known about the molecular

mechanisms by which TBX3 is up-regulated in melanomas, $BRAF^{V600E}$ elevates TBX3 transcription which in turn potently represses E-cadherin expression in both primary melanocytes and melanoma cells [140,141].

The key role played by *TBX3* in promoting invasion/metastasis was reinforced by the observation that it also promotes migration of MCF-7 breast cancer cells [67]. *TBX3* is directly up-regulated by c-Jun and JunB which enhances protein kinase C (PKC) signalling to promote cell migration of the MCF-7 breast cancer cells [142]. Similarly, Du et al. [143] confirmed that the PKC–TBX3–E-cadherin signalling cascade also operates in human bladder cancer cell lines. TBX3 also plays a pivotal role in mediating the anti-proliferative and pro-migratory role of TGF-β1 in breast epithelial and skin keratinocytes. Treatment of these cells with TGF-β1 up-regulates TBX3 protein and mRNA levels through Smad3/4 and JunB cooperatively binding the TBX3 promoter [144]. TBX3's role in cancer cell migration and invasion was further highlighted by work performed in head and neck squamous cell carcinoma cell lines in which TBX3 is up-regulated. Importantly, silencing TBX3 in these cells reduced their invasiveness and induced anoikis, a specific form of cell death induced by anchorage-dependent cells detaching from the surrounding extracellular matrix [125].

A recent study by Fillmore et al. [145] has also suggested that TBX3 may contribute to oncogenesis through promoting the expansion of breast cancer stem cells (CSCs). When TBX3 was silenced in the MCF-7 breast cancer cells the formation of an oestrogen-induced CSC population was abrogated while the overexpression of TBX3 was sufficient to expand the number of CSCs. The molecular pathway involved oestrogen stimulation of fibroblast growth factor 9 (FGF9) and its receptor (FGFR3) that resulted in TBX3 activation. The potential role of TBX3 in regulating stemness or pluripotency was recently revealed by Zhao et al. [146] who showed that the Tbx3 isoforms, Tbx3 and Tbx3 + 2a, are highly expressed in mouse embryonic stem cells (mESCs) and play essential roles in maintaining pluripotency. Ectopic overexpression of either of the two isoforms could induce mESCs to differentiate by a mechanism involving the inhibition of Nanog, a key pluripotency factor. Interestingly, while Tbx3 + 2a appeared to directly repress Nanog, the mechanism by which Tbx3 does so appears to be indirect. Whether the role of Tbx3 in stem cell regulation impacts on its role in cancer is unresolved and requires a better characterisation of its target genes.

In contrast to the significant body of research implicating TBX3 as a tumour promoter, there is also some evidence that TBX3 may act as a tumour suppressor in certain cellular contexts. In a microarray study, Lyng et al. [134] showed that TBX3 levels were down-regulated in uterine and cervical cancer samples positive for lymph node metastases and that TBX3 expression correlated with an increase in progression-free survival. In addition, TBX3 is epigenetically silenced by methylation in glioblastoma, bladder and gastric cancer (Fig. 3) [147–149]. *TBX3* methylation in bladder cancer was associated with the progression of non-muscle invasive tumours (Pta) to muscle-invasive tumours (MI) and patients in which *TBX3* was not methylated had significantly better progression-free survival rate [147]. In addition, TBX3 methylation was associated with a significantly lower survival rate in a cohort of glioblastoma patients [148], and while genomic screening identified TBX3 as a methylated gene in at least one gastric cancer cell line [149], the possibility that this may be related to the development and progression of gastric cancers has not yet been investigated. These studies are consistent with the notion that TBX3 may have a tumour-promoting or a tumour-suppressing role in cancers of different cellular origins and contexts.

2.3. Brachyury

Brachyury (*T*) is the proto-type of the T-box gene family with a well-defined role in mesoderm specification and recent evidence suggests that it may have tumour promoting and tumour suppressor properties. It is overexpressed in several tumours of epithelial origin, including cancers of the oesophagus, stomach, small intestine, kidney, bladder, uterus, ovary, testis and lung [150] and duplication of the Brachyury gene has been associated with chordoma, a rare bone cancer [151]. Importantly, a number of studies have implicated Brachyury, like TBX3, in promoting EMT, an important step during metastasis [19,96,152–155]. Fernando et al. [19] first showed that the expression of Brachyury results in EMT using overexpression and knockdown models together with an in vivo mouse model. The authors showed that an increase of Brachyury expression in several cancer cell lines lead to EMT as determined by an increase in the expression of the mesenchymal markers, fibronectin, N-cadherin, and vimentin and a loss of the epithelial markers, E-cadherin and plakoglobin. Consistent with this, silencing Brachyury inhibited EMT in these cells and when nude mice were subcutaneously injected with control or *Brachyury* knockdown metastatic lung cancer cells, tumours from the knockdown cells had much lower levels of mesenchymal markers. Furthermore when these cells were injected intravenously into nude mice, 60% of mice bearing control tumours developed lung metastases, which was the case for only 18.8% of mice bearing *Brachyury* knockdown tumours. More recently it was shown that Brachyury is a mediator of TGF-β1 induced EMT and that its overexpression correlates with resistance to anti-cancer therapy [156,157]. Huang et al. [156] showed in vitro and in vivo that lung cancer cells with high levels of Brachyury had a significant survival advantage when treated with radiation therapy and conventional chemotherapeutic agents than cells expressing low levels of the protein. As expected, the overexpressing cells proliferated slower although it was demonstrated that Brachyury directly represses the tumour suppressor *p21*, which may provide a molecular mechanism for the observed resistance. In addition to its role in EMT, Shimoda et al. [155] and Sarkar et al. [154] demonstrated that *Brachyury* may also confer stem cell characteristics on cancer cells. For example, silencing *Brachyury* reversed the sphere-forming ability and tumorigenicity of adenoid cystic carcinoma cells [155] and regulated cancer stem cell markers such as *NANOG* [154]. While the above studies point to Brachyury promoting cancer, Park et al. [18] suggested that it plays a tumour suppressor role in lung cancer. Analysis of a microarray used to determine differential promoter methylation in two lung cancer cell lines which were each treated with or without a demethylation agent revealed that *Brachyury* was among the upregulated genes, suggesting that it was originally methylated in these cells. Experiments measuring mRNA levels confirmed that *Brachyury* is silent in the majority of lung cancer cell lines and lung adenocarcinoma tissues tested [18]. While these results suggest a correlation between *Brachyury* gene silencing and lung cancer, it remains to be proven whether this is a direct cause of lung cancer.

2.4. Eomes

Eomes, a member of the T-brain1 subfamily, has also been implicated as a tumour suppressor. Eomes plays a critical role in immunity and defence by activating effector CD8 + T cells [158,159] and there is evidence that these T cells play an important role in colorectal cancer and anti-tumour immunity [160]. This prompted investigations into the potential role of Eomes in colorectal cancer. Indeed, an inverse relationship between Eomes expression and the presence of lymph node metastases in colorectal cancer was observed [16]. In situ hybridisation and RT-PCR confirmed that this relationship was unique, as a similar correlation was not observed with any other transcription factor associated with CD8 + T cells. Lastly, the mechanism through which Eomes reduces lymph node metastasis was shown to involve the activation of CD8 + T cells via perforin induction.

2.5. TBX1

Tbx1 is required for inner ear [161] and heart development [162], and has been shown to play a role in the regulation of heart progenitor

cell proliferation and differentiation [162]. Furthermore, *Tbx1* was identified as a key gene in DiGeorge syndrome (DGS) after homozygous null mice exhibited the primary features of DGS, including thymus/parathyroid hypoplasia, craniofacial abnormalities, cleft palate, and abnormalities in the cardiac outflow tract [163]. *Tbx1* is also uniquely expressed in adult hair follicle stem cells, and regulates progenitor cell proliferation and differentiation in development. This led Trempus et al. [15] to perform experiments to determine the contribution of *Tbx1* to mouse skin tumour development. In their initial studies, they observed that while *Tbx1* was expressed in skin stem cells, expression was not detected in benign papillomas or various cutaneous malignancies which suggested that Tbx1 may have tumour suppressor activities in these tumours [15]. To test this possibility, the authors ectopically expressed Tbx1 in a highly tumourigenic mouse spindle carcinoma cell line, which lacks endogenous Tbx1, and examined the tumour forming ability of the resulting cell lines in mice. They show that Tbx1 expression significantly suppressed tumour growth. Furthermore, in culture, ectopic Tbx1 expression resulted in decreased cell growth and reduced ability to form multi-layered colonies compared to control cells, with Tbx1-expressing cells accumulating predominantly in G1 phase. Lastly Trempus et al. [15] showed that contact inhibition could be restored by expressing Tbx1, which caused a dose-dependent decrease in cell proliferation. Thus Tbx1 may promote a senescent or quiescent cell cycle profile, the opposite of what is seen with TBX2 or TBX3, which may be consistent with TBX1 activating similar sets of target genes to those that are repressed by TBX2 and TBX3.

2.6. TBX4

TBX4 is involved in the regulation of hindlimb outgrowth and specification during embryonic development [22,164] and while not previously implicated in the development of the pancreas and liver, recent studies have shown that it may act as a tumour suppressor in pancreatic ductal cell adenocarcinoma (PDAC) and intrahepatic cholangiocarcinoma. Proteomic analysis identified *TBX4* as a biomarker for PDAC, because low *TBX4* levels could be correlated with tumour stage, increased metastasis and a decrease in overall patient survival [165,166]. Similarly TBX4 was identified as a biomarker and predictor of tumour stage in intrahepatic cholangiocarcinoma, however clinicopathological characteristics of patients could not be associated with *TBX4* expression [166].

2.7. TBX5

Tbx5 is exclusively expressed in the developing forelimb and mutations in human TBX5 cause Holt–Oram Syndrome (HOS), a dominant disorder characterised predominantly by upper (fore) limb defects and heart abnormalities. Even though there is no current evidence that TBX5 contributes to colon development, a very comprehensive study by Yu et al. [14] described *TBX5* as a potent tumour suppressor in colon cancer. Compared to normal tissue, *TBX5* was silenced by promoter methylation in colon cancer cell lines and this was validated in primary colon tumours. Importantly, colon cancer patients with *TBX5* promoter methylation showed a significantly lower overall survival. When *TBX5* was re-expressed in colon cancer cell lines, key features of the cancer phenotype such as increased proliferation, substrate independence and increased cell migration, were reversed and apoptosis was induced via caspases 3 and 7 and poly (ADP-ribose) polymerase (PARP) [14]. The tumour suppressor role for TBX5 was challenged in a later study in which TBX5 was shown to be involved in the activation of anti-apoptotic genes in a colon cancer cell line [167]. The authors showed that in β-catenin driven cancer cell lines, including several derived from colon cancers, TBX5 forms a complex with YAP1 and β-catenin, which localises to the promoters of the anti-apoptotic genes *BCL2L1* and *BIRC5*.

2.8. T-bet (TBX21)

T-bet is exclusively expressed in the olfactory bulb and the thymus of the developing mouse [168]. The thymus plays a key role in expressing Oestrogen receptors (ER) in both males and females [169] and T-bet is also overexpressed in cancers that are hormone dependent. For example, T-bet is overexpressed in a subset of ER alpha (ERα)-positive breast cancers where it may function to promote tumourigenesis [21]. McCune et al. [21] investigated the role that insulin may play in the hormonal network of ERα positive breast cancers and showed in MCF-7 breast cancer cells that insulin treatment led to the induction of T-bet. Increased expression of T-bet resulted in a disrupted oestrogen receptor hormonal network leading to resistance to hormone therapy and an unfavourable prognosis. This network includes the transcription factors Gata3 and FoxA1 whose expression was found to be down-regulated following insulin treatment or stable overexpression of T-bet. Furthermore, T-bet overexpressing cells were also more resistant to tamoxifen therapy in the presence of insulin and displayed elevated ERK and AKT activation, which is associated with anti-oestrogen resistance.

3. Concluding remarks and unresolved questions

A developing embryo and a growing tumour share many common features including fast proliferation and migration. It is therefore not surprising that important developmental regulators such as the T-box family members have been implicated in driving cancer progression. Interestingly, the roles of T-box factors in cancer often, but not always, parallel with their functions during embryogenesis. For example, consistent with TBX2 functioning as a pro-proliferative factor in several cancers, in part by repressing p21, it has also been shown to promote proliferation of fibrocytes and myoblasts during lung development and myogenesis respectively by inhibiting this cyclin dependent kinase inhibitor [170,73]. Furthermore, Tbx2 is critical for the development of the chick ventral tubero-mamillary (vt-m) hypothalamus by its ability to repress sonic hedgehog (shh) in floor plate-like cells which is key for their progression to proliferating vt-m hypothalamic progenitors [171]. In the developing mammary gland, however, Tbx2 has no effect on p21 levels [172] and it inhibits cell proliferation during cardiogenesis, in part, by repressing N-myc [173,174]. It would therefore appear that the effect of TBX2 on proliferation will depend on the cellular context which may be determined by the availability of specific co-factors. We predict that this will be the case for the roles of most T-box factors and that the relationship between their role(s) in embryonic development and in cancer can only be resolved once the repertoire of their target genes and co-factors are fully characterised.

To date the T-box factors have primarily been implicated in regulating senescence, and invasion and metastasis, with the latter perhaps being related to a more general phenomenon of promoting or stabilizing a more stem-like state. While the opposing actions of some family members in these processes may reflect differing abilities to regulate transcription — for example, TBX2 and TBX3 as transcription repressors and TBX1 as a transcription activator — it is likely that this will turn out to be too simplistic. For other transcription factors it is clear that the capacity to activate or repress transcription can be gene or cell type-dependent, and it seems likely that each T-box factor may activate or repress transcription in different contexts. Moreover, while a few target genes have been identified that are likely implicated in the regulation of proliferation and invasiveness, most notably the cyclin-dependent kinase inhibitors, E-cadherin and PTEN, the full repertoire of the great majority of T-box factor target genes have yet to be identified. In this respect, we do not know how different the sets of genes targeted by co-expressed T-box factors might be. Indeed identifying potential target genes based on the presence of the consensus AGGTGTGA T-element may be misleading. T-box factors in general have an interesting mode of DNA recognition that suggests they will make use of cooperating

DNA binding proteins to regulate gene expression. Given the high homology within the T-domain, the DNA-co-crystal structures of Brachyury [175], TBX3 [176] and TBX1 [177] reveal that these factors, and by implication all T-box factors, recognize DNA via an alpha-helix inserted into the minor groove of the DNA. Since in normal B-DNA, the minor groove is not wide enough to accommodate an alpha-helix, the minor groove found in complex with the T-box factors is widened. Moreover only two base-specific DNA contacts are made, with the third and fifth bases of the AGGTGTGA consensus recognition motif. These observations have several implications.

Firstly, two base specific contacts GXG, are insufficient to provide DNA-binding specificity, implying that T-box factors must use interactions with other DNA binding proteins to achieve any degree of target specificity. Consistent with this, Brachyury for example can bind in a complex with Smad1 [178], while Tbx2 and Tbx5 can use the homeodomain protein NKX2.5 [179,180]. Whether additional DNA-binding factors cooperate with TBX2 and TBX3 is unknown, but it seems likely given the observation that TBX2 repression of the NDRG1 and CST6 genes are mediated via EGR1 (Krox-24) [72], a zinc-finger transcription factor, raising the possibility that TBX2 and EGR1 act as a repressor complex on some genes. It is also possible that binding in the minor groove will leave the opposing major groove free for recognition by a cooperating DNA-binding factor, effectively clamping the DNA between the two proteins to provide a high affinity DNA-binding complex.

Secondly, the consensus sequence identified using in vitro binding site selection is unlikely to be correct in vivo, though it may be bound in some cases. Rather it seems likely that in part this sequence was selected because an alternating purine pyrimidine stretch may have a natural predisposition to having a wide minor groove [181] that would facilitate T-box factor binding. Moreover, in the minor groove C and T-residues would appear identical to a DNA-binding protein. As such there is no reason to suppose that in vivo the T and C-residues would not in some cases be substituted, unless a cooperating factor is needed to recognize specific residues in the major groove.

Third, in vivo the presence of a natural wide minor groove, as is found on DNA wrapped around nucleosomes, would facilitate DNA binding. Consistent with this, TBX2 can bind nucleosomal DNA and its binding is regulated by the presence of the histone tails [76]. These data suggest that one role of T-box factors may be to act as pioneer transcription factors acting to bind nucleosomes and open up chromatin, as has been seen in vivo for T-bet [182] and TBX2 [76] that can convert closed heterochromatin to more open euchromatin, a property that would be invaluable in mediating cell fate switches. Alternatively repressive T-box factors like TBX2 and TBX3 could fix a nucleosome in position to repress transcription and prevent access of either activating transcription factors or the basal transcription machinery. This would be especially relevant for genes, such as p21 [58,130] and TYRP1 [24,183], where binding to the transcription initiator has been seen. Until the genome-wide occupancy of TBX2 and TBX3 are determined, the precise mechanism by which they regulate gene expression and their relationship to nucleosome positioning will remain obscure.

Nevertheless it is clear that the analysis of the role and regulation of T-box factors in cancer presents some interesting therapeutic opportunities. Work in a number of cancer types has shown that silencing T-box factor expression can reactivate intact, but suppressed senescence pathways. Since pro-proliferative oncogenes provide a powerful pro-senescence signal, therapies designed to manipulate T-box factor function in cancer may facilitate pro-senescence therapy without substantial effects on non-cancer cells that do not harbour oncogenic mutations. Although directly targeting T-box factors is unlikely to be a viable option three approaches can be envisaged alone or in combination: targeting signalling pathways required for T-box factor expression; targeting the enzymatic activities of T-box factor co-factors; and targeting the signalling pathways that regulate the interactions between T-box factors and their co-factors. Although substantially more insights are needed before this can be a viable approach, the restoration of senescence in some cancers on T-box factor inactivation strongly suggests that it is worth pursuing.

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