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Original Articles

COL1A2 is a TBX3 target that mediates its impact on fibrosarcoma and chondrosarcoma cell migration

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ABSTRACT

The developmentally important T-box transcription factor TBX3, is overexpressed in several cancers and contributes to tumorigenesis as either a tumour promoter or tumour suppressor. For example, TBX3 promotes cell proliferation, migration and invasion of chondrosarcoma cells but inhibits these processes in fibrosarcoma cells. This suggests that the cellular context influences TBX3 oncogenic functions, but the mechanism(s) involved has not been elucidated. *COL1A2* encodes type I collagen and, like TBX3, plays important roles during embryogenesis and can act as either oncogene or tumour suppressor. Here we explore the possibility that COL1A2 may be a TBX3 target gene responsible for mediating its opposing oncogenic roles in chondrosarcoma and fibrosarcoma cells. Results from qRT-PCR, western blotting, luciferase reporter and chromatin immunoprecipitation assays show that TBX3 binds and activates the *COL1A2* promoter. Furthermore, we show that TBX3 levels are regulated by AKT1 and that pseudo-phosphorylation of TBX3 at an AKT consensus serine site, enhances its ability to activate COL1A2. Importantly, we demonstrate that COL1A2 mediates the pro- and anti-migratory effects of TBX3 in chondrosarcoma and fibrosarcoma cells respectively. Our data reveal that the AKT1/TBX3/COL1A2 axis plays an important role in sarcomagenesis.

1. Introduction

Haploinsufficiency of TBX3, a member of the developmentally important family of T-box transcription factors, is responsible for the autosomal dominant human ulnar-mammary syndrome (UMS), which is characterized in part by limb malformations and mammary gland aplasia [1–3]. On the other hand, the overexpression of TBX3 has been reported in several carcinomas where it directly promotes tumorigenesis through multiple mechanisms. TBX3 can behave as an anti-senescence and pro-proliferative factor by directly repressing the negative cell cycle regulators $p14^{ARF}$ and $p21^{WAF1}$ [4–7] and contributes to tumour formation, invasion and metastasis [8]. Recently, TBX3 was also reported to be overexpressed in a diverse subset of soft tissue and bone sarcoma [9]. However, while promoting cell proliferation, migration and invasion of chondrosarcoma, rhabdomyosarcoma and liposarcoma cells in vitro and in vivo, TBX3 inhibited these processes in fibrosarcoma cells [9]. There is however nothing known about what regulates these dual roles of TBX3.

The extracellular matrix (ECM) is an essential component of the tumour microenvironment. Indeed, cancer development and progression are associated with increased ECM deposition and signals elicited from the ECM are necessary for cancer cell proliferation and invasion [10]. Type I collagen, a triple helix composed of two COL1A1 and one COL1A2 chains, is the most abundant component of the ECM [11]. Changes in the synthesis of type I collagen occur in wound healing, during embryogenesis and in certain pathological conditions, such as cancer, scleroderma, and fibrosis of the liver, lung and kidney [12]. In chondrosarcoma and osteoblastoma, transcription factors such as Sox9 and Runx2 respectively have been described as regulators of cartilage matrix genes which include type 1 collagen which play important roles in the structure and function of cells [13]. Like TBX3, collagen can be a double-edged sword in the cancer process where it can both inhibit and promote tumour progression. Indeed, there is evidence that COL1A2 plays a role in medullablastoma and melanoma development and angiogenesis and that it contributes to invasion and metastasis of melanoma and gastric carcinoma [14–17]. Conversely, COL1A2 can inhibit cell proliferation, migration and invasion of colorectal [18] and bladder [19] cancer cells; can decrease in vitro colony forming ability of liver epithelial cells [20]; and can suppress the tumour forming ability of transformed fibroblast cell lines in vivo

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[21–23]. Furthermore, COL1A2 levels significantly decrease upon cellular transformation of fibroblasts [22,24,25] and *COL1A2* is methylated in a number of human cancer cell lines including fibrosarcoma [18,19].

The AKT pathway is constitutively active in many human cancers including various soft tissue sarcomas and is known to play a key role in tumorigenesis [26–28]. AKT is a serine/threonine kinase which exists as three isoforms viz AKT1, AKT2 and AKT3 and while highly homologous these isoforms are differentially expressed in different cancers and can be functionally distinct [29]. Through phosphorylation of a range of substrates, the AKT isoforms regulate a variety of cellular processes including proliferation, survival, motility, angiogenesis and glucose homeostasis [30,31]. Interestingly, AKT3 phosphorylates TBX3 at serine 720 and this stabilizes the protein leading to its overexpression in advanced melanoma cells [32]. Furthermore, AKT3-phosphorylation of TBX3 promotes its nuclear localisation and enhances its ability to repress the cell cycle adhesion molecule E-cadherin and promote migration in melanoma [32]. The AKT signalling pathway has also been implicated in the upregulation of *COL1A2* expression but the transcription factor(s) responsible for this has not been identified [33–35].

The present study shows that TBX3 directly binds and transcriptionally activates *COL1A2* gene expression in transformed fibroblasts and chondrosarcoma cells. We reveal that pseudo-phosphorylation of TBX3 at a highly conserved serine proline motif in its DNA binding domain can inhibit this transcriptional regulation. Furthermore, we show that AKT1 upregulates TBX3 levels and that pseudo-phosphorylation of TBX3 at the AKT motif at S720 enhances its ability to activate *COL1A2* in fibrosarcoma and chondrosarcoma cells. Importantly, we demonstrate that while COL1A2 mediates the inhibitory effect of TBX3 on fibrosarcoma cell migration, it mediates, in part, the TBX3-induced migration of chondrosarcoma cells.

2. Materials and methods

2.1. Cell lines and culture conditions

Gamma-radiated transformed WI-38 human lung fibroblast cells (CT-1) [36] and SW1353 human chondrosarcoma cells (ATCC HTB-94) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Highveld Biological, South Africa), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100U/ml penicillin and 100μg/ml streptomycin at 37 °C (95.0% air/5% CO^2, 65% humidity). CT-1 and SW1353 cells in which TBX3 was stably knocked down using a pSuper.neo/GFP expression vector containing a sequence targeted to TBX3 or a nonspecific control was previously established and cultured as described [8,39].

2.2. Cell treatments

Cells were seeded at a density of 1.4×10^5 cells in a 6 well plate and serum starved (DMEM containing 1% FBS) for 24 h after which they were treated with either vehicle (DMSO) or 10 μM AKTVIII inhibitor (Calbiochem, USA) for 1, 2 and 4 h.

2.3. Plasmid constructs

The human *COL1A2* promoter luciferase reporter constructs were described previously [37]. The human pCMV-TBX3-HA expression construct was kindly provided by Dr Christine Campbell of the Cleveland Clinic Foundation, USA. The HA-tagged human pCMV-TBX3 DNA-binding mutant (DBM), pCMV-TBX3 N-terminal, pCMV-TBX3 S190A and pCMV-TBX3 S190E mutant expression constructs have previously been described [7]. The FLAG-tagged mouse pCMV-mtbx3, pCMV-mtbx3 + 2a and pCMV-mtbx3-DBM constructs were kindly provided by Professor Colin Goding (Ludwig Institute of Cancer Research, UK). The HA-tagged human pCMV-TBX3 S720A and S720E mutant expression constructs have previously been described [38].

2.4. Site-directed mutagenesis of the COL1A2 promoter luciferase construct

Site-directed mutagenesis (SDM) was performed with the 2.4 kb COL1A2 promoter-luciferase reporter construct as a template using KAPA HiFi HotStart ready mix (KAPA Biosystems, South Africa) and the following primers:

MUT -2328: 5′-GC CCTGCAAAGGTAATTCAGCACAGaatTcACAATG ATTCTTAG -3′; MUT -2190 5′-GTTAGTTCTGTAACGGaatT cTTTCAGG GAAATGTTAAAAATG-3′; MUT -1314 5′-GTGGGCGCACTGCTTG GGAtAtCaCCAGCGAAGACT ACGAAG-3′; MUT -1258 5′-GAAGGGCCT CTGGATGaatTcGGGGAGGTGCTTGGGTG-3′; MUT -1005 5′-GG TTGG AGTCGTGTCGGAGaattcGACCATCCCCCAAAAGACCC-3′; MUT -875 5′-ATCCTCCCTGTAGCCGGcTag CAAGCAGCCTCGAGCC-3′.

2.5. Transfections and luciferase assays

Transient transfections were performed using FuGENE®HD (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. CT-1 and SW1353 cells were plated at 7×10^4 cells per well of a 12-well plate 24 h before transfection. 200 ng of the relevant *COL1A2* reporter plasmid and varying amounts of the TBX3 expression construct or empty vector were transfected. 10 ng of the RL-CMV vector (Promega, Madison, WI, USA), which contains the cytomegalovirus promoter driving the expression of a renilla reporter, was used as an internal control for transfection efficiency. Cells were cultured for 30 h, and extracts assayed for firefly and Renilla luciferase activity using the Dual-Luciferase Reported Assay System (Promega) and a Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA, USA). Firefly luciferase values were expressed relative to empty-vector control.

2.6. siRNA sequences and transfection

Transient knockdown of AKT1 and COL1A2 expression was achieved using siRNA (small interfering RNA). Cells were transfected with 10 nM anti-AKT1 siRNA (L-003000-00-0005; Dharmacon, Lafayette, CO, USA), 5 nM anti-COL1A2 siRNA (L-003000-00-0005; Dharmacon, Lafayette, CO, USA), or a control (non-silencing)siRNA (Qiagen, USA), using Lipofectamine LTX (Invitrogen Life Technology, San Diego, CA, USA) according to the manufacturer's instructions.

2.7. Western blot analysis

Cells were harvested and protein prepared as described previously [39]. Primary antibodies used were as follows: rabbit polyclonal anti-TBX3 (1:500; 42-4800; Zymed or 1:1000; 99302; Abcam) and polyclonal rabbit anti-p38 (1:10000; M0800; Sigma-Aldrich), monoclonal mouse anti-HA (1:1000; H9658; Sigma-Aldrich), mouse monoclonal anti-FLAG M2 (1:1000; F1804; Sigma-Aldrich), goat polyclonal anti-COL1A2 (1:500; sc-8786; Santa Cruz), rabbit polyclonal anti-phospho AKT (Ser473) (1:1000; 9271; Cell Signalling), rabbit polyclonal anti-AKT1 (1:1000; C73H10; Cell signalling), mouse monoclonal anti-N-Cadherin (1:1000; 13A9; Cell Signalling), rabbit monoclonal anti-β-Catenin (1:1000; D10A8; Cell Signalling) and rabbit polyclonal anti-Vimentin (1:1000; R28 Cell Signalling). Signal was detected using peroxidase-conjugated goat anti-mouse or anti-rabbit (1:5000, Biorad) or donkey anti-goat (1:5000; Santa Cruz) secondary antibodies and visualised by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using the high pure RNA isolation kit (Roche, Germany) according to the manufacturer's

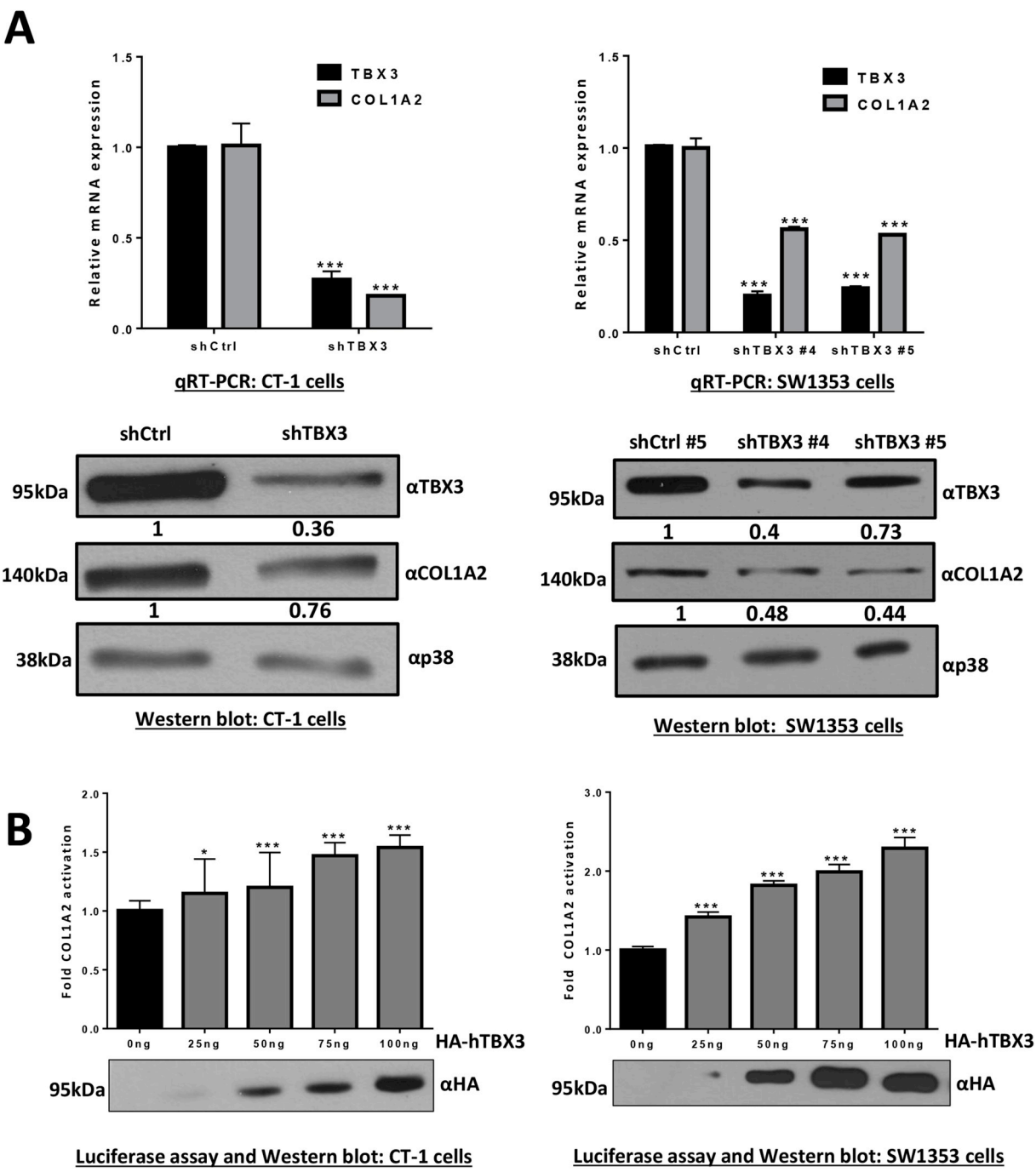
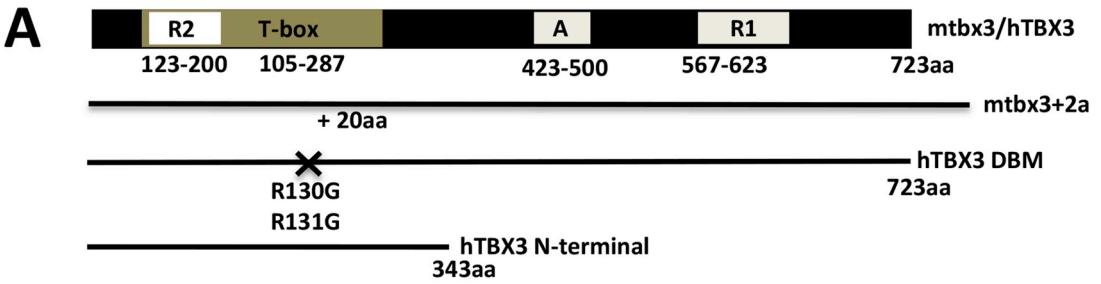
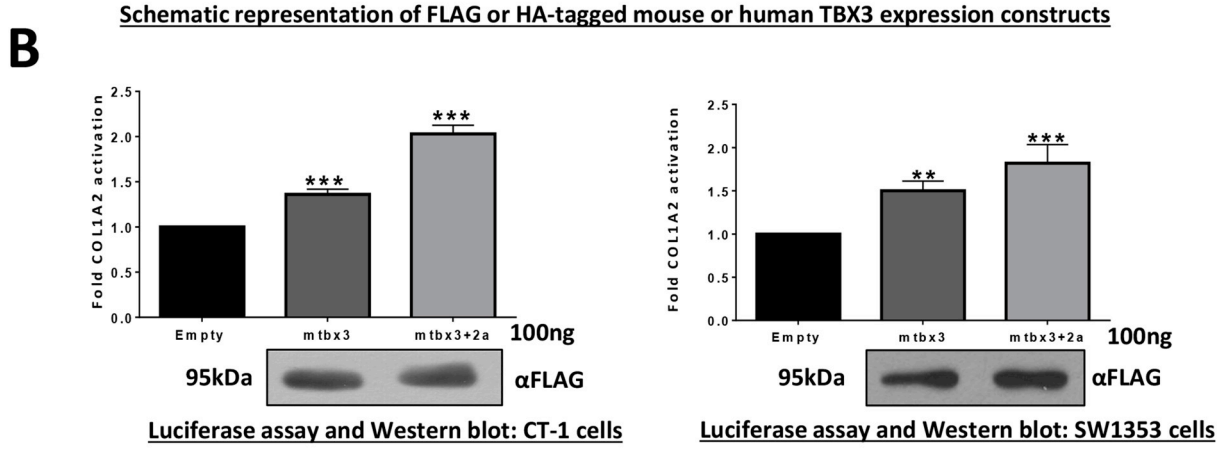


Fig. 1. TBX3 is a direct transcriptional activator of *COL1A2* expression. A. Total RNA and protein were extracted from CT-1 transformed fibroblasts and SW1353 chondrosarcoma control and TBX3 knockdown cells. *Upper panels*: quantitative real-time PCR was performed on reverse transcribed RNA using primers specific to TBX3 and COL1A2 and mRNA levels were normalised against GUSB levels. *Lower panels*: protein extracts were subjected to western blot analysis with antibodies specific to TBX3 and COL1A2. Densitometric readings were calculated relative to the p38 loading control. **B.** CT-1 and SW1353 cells were co-transfected with the *COL1A2* promoter luciferase reporter construct and varying amounts of the HA tagged human TBX3 expression construct. Total amount of plasmid DNA transfected was held constant using the corresponding HA-Empty vector. The pCMV renilla luciferase reporter plasmid was used to control for transfection efficiency. Luciferase activity was measured and data were normalised against renilla values and fold activation of the *COL1A2* promoter was calculated relative to the empty vector control. *Lower panels*: western blot analyses show expression of the HA tagged TBX3 using an HA antibody. **A, B.** The values in the graphs represent the mean of three independent experiments ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001).

instructions and reverse transcription performed using 1 μg of RNA and the InProm-II™ reverse transcription system (Promega A3800, USA) according to the manufacturer's instructions. The reactions were carried out using 2 μl of cDNA, Power SYBR® Green PCR Master Mix (Applied Biosystems, UK) and primers to amplify the human *TBX3* (QT00022484; Qiagen), *COL1A2* (QT00072058, Qiagen), *AKT1* (5′-ATGAGCGACGTGGCTATTGTGAAG-3′ and 5′-GAGGCCGTCAGCCAC AGT CTGGATG-3′), *AKT2* (5′-TGCTTGAGGCTGTTGGCGACC-3′ and 5′-ATGAATGAGGTGTCTGTCATCAAAGAAGGC-3′) and *AKT3* (5′-ATG AGCGATGTTACCATTGT-3′ and 5′-CAGTCTGTCTGCTACAGCCTGG ATA-3′) (Integrated DNA Technologies) and *GUSB* (QT00046046; Qiagen). qRT-PCR was performed using the StepOnePlus™ PCR system (Applied Biosystems) with the following parameters: denaturation for 15 min at 95 °C and combined annealing and extension for 35 cycles at

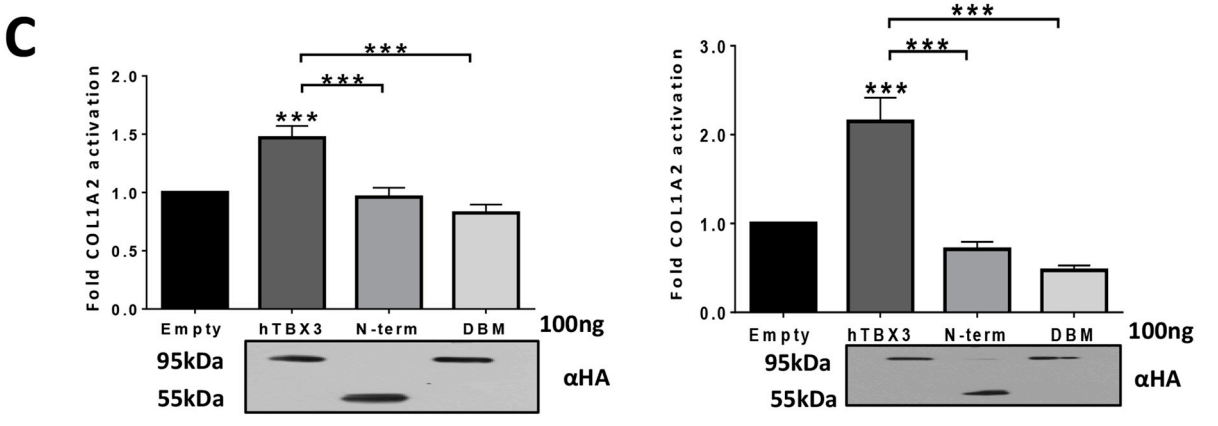


Schematic representation of FLAG or HA-tagged mouse or human TBX3 expression constructs



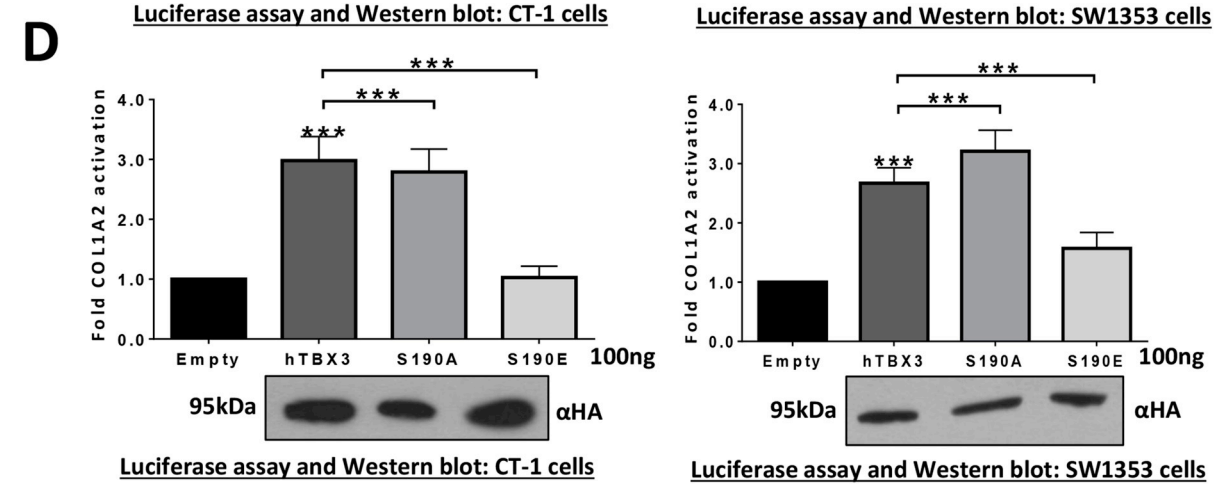
Luciferase assay and Western blot: CT-1 cells

Luciferase assay and Western blot: SW1353 cells



Luciferase assay and Western blot: CT-1 cells

Luciferase assay and Western blot: SW1353 cells



Luciferase assay and Western blot: CT-1 cells

Luciferase assay and Western blot: SW1353 cells

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Fig. 2. The DNA binding and C-terminal domains of the TBX3 protein are required for activation of *COL1A2*. **A.** Schematic representation of expression constructs for: human and mouse wild type TBX3 (hTBX3 and mtbx3 respectively), mouse tbx3 + 2a (mtbx3 + 2a) isoform which contains an additional 20 amino acids within the DNA binding domain, the hTBX3 DNA binding mutant in which the indicated residues have been mutated (hTBX3 DBM) and a truncated hTBX3 N-terminal (hTBX3 N-term). "A" and "R" denote activation and repression domains respectively. **B.** CT-1 transformed fibroblasts and SW1353 chondrosarcoma cells were co-transfected with the *COL1A2* promoter luciferase reporter construct together with either the mtbx3 or mtbx3 + 2a expression constructs. Western blot analysis with an antibody to FLAG shows expression of FLAG tagged mtbx3 and mtbx3 + 2a. **C.** CT-1 and SW1353 cells were co-transfected with a *COL1A2* promoter luciferase reporter construct together with pCMV Empty, hTBX3, hTBX3 N-term or hTBX3 DBM expression constructs. Western blot analysis with an HA antibody shows expression of HA tagged WT hTBX3, hTBX3 N-term, hTBX3 DBM. **C**. CT-1 and SW1353 cells were co-transfected with the *COL1A2* promoter luciferase construct together with HA-tagged pCMV Empty, hTBX3, hTBX3 S190A or hTBX3 S190E expression vectors. **B, C, D.** The pCMV renilla luciferase reporter construct was used to control for transfection efficiency. Cells were lysed thirty hours post transfection and luciferase activity measured. Fold activation of *COL1A2* promoter activity was calculated relative to that of the Empty pCMV vector. The values indicate the mean of three independent experiments ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

60 °C for 1 min. Samples were prepared in triplicate and non-template controls were included to detect contamination or non-specific amplification. The $2^{-\Delta\Delta Ct}$ method was employed to analyse results and relative mRNA expression levels of TBX3 and COL1A2 were normalised to mRNA levels of glucuronidase-beta (GUSB).

2.9. Chromatin immunoprecipitation assay (ChIP)

ChIP assays were carried out as previously described [7]. Briefly, CT-1 and SW1353 cells were fixed in 1% formaldehyde and the chromatin extracted, sonicated, and immunoprecipitated using antibodies against TBX3 (8 µg; sc-17871, Santa Cruz Biotechnology Inc) or IgG (8 µg; sc-2027, Santa Cruz Biotechnology Inc). DNA precipitated was analysed by qRT-PCR using human COL1A2–specific primer pairs (Forward-5′-GCCCTGCAAAGGTAATTCAGCAC-3′, Reverse-5′-CAACAA CAAAAGACCTGAGGTTGA-3′) or a nonspecific GAPDH promoter region (Forward-5′- CAGCCAGACGAGGACACA-3′, Reverse-5′-CCTTTCT GGGATTGCCTTTC-3′). Crossing values (Ct) of TBX3 and IgG precipitated DNA were adjusted by normalising against the Ct value of 1% of input DNA and the ΔΔCt method was used to determine fold enrichment. The equation was used as follows: $2^{-(\Delta Ct1 - \Delta Ct2)}$ (ΔCt1 = TBX3, ΔCt2 = IgG).

2.10. In vitro cell migration assay

Cell migration in culture was measured using a two-dimensional in vitro scratch motility assay as previously described [40]. Briefly, CT-1 and SW1353 cells were transfected with siControl or siCOL1A2 for 24 h before being replated at 100% confluency. The wound areas were measured over a period of 9 h using ImageJ software (National Institutes of Health, Bethesda, MD) [41].

2.11. Statistical analysis

Statistical significance was determined using the Student's t-test (Excel, Microsoft, Redmond, WA). Significance was accepted at $p < 0.05$.

3. Results

3.1. TBX3 is a direct transcriptional activator of COL1A2

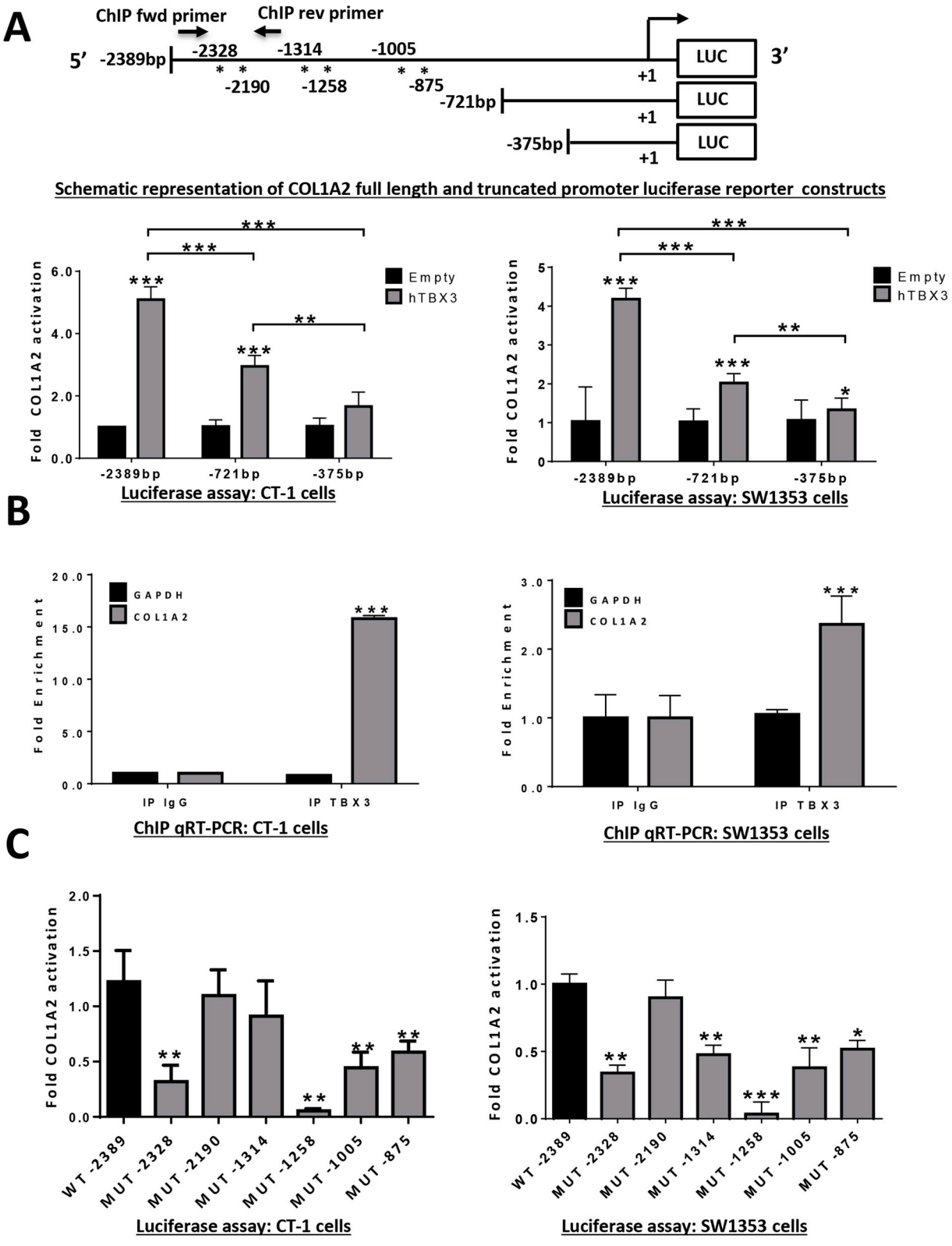
TBX3 was previously shown to promote tumorigenesis in chondrosarcoma but to inhibit several aspects of the cancer phenotype in fibrosarcoma [9]. We were thus keen to identify TBX3 target gene(s) that could explain these opposite functions. In this regard we were interested in reports that COL1A2 can function as either tumour promoter or tumour suppressor. We therefore wanted to establish whether TBX3 can regulate *COL1A2* in fibrosarcoma and chondrosarcoma cells and how this regulation may impact tumorigenesis. To this end, we firstly compared the COL1A2 mRNA and protein levels in transformed CT-1 fibroblasts and SW1353 chondrosarcoma cells in which TBX3 was stably knocked down (shTBX3) to that of control cells (shCtrl). It is important to note that while two different SW1353 clones (shTBX3 #4 and #5) were included in our analyses, it was technically difficult to obtain individual CT-1 shTBX3 clones and therefore pooled CT-1 shTBX3 cells were used. Throughout this study, p38 was used as a loading control in western blot analyses because although its activity is modulated by phosphorylation, total levels of this protein remain unchanged during the cell cycle and in response to stimuli [42]. Quantitative real time PCR (qRT-PCR) and western blotting show that COL1A2 mRNA and protein levels were significantly downregulated in CT-1 shTBX3 and SW1353 shTBX3 cells (Fig. 1A) revealing a direct correlation between TBX3 and COL1A2 levels.

We next performed luciferase reporter assays and demonstrate that TBX3 significantly activated the *COL1A2* promoter in a dose-dependent manner in CT-1 and SW1353 cells (Fig. 1B). Lysates used for the luciferase assays were also subjected to western blotting and results showed that increasing amounts of transfected TBX3 protein were achieved (lower panel of Fig. 1B). Alternative splicing of the TBX3 gene results in the TBX3 and TBX3 + 2a isoforms with TBX3 + 2a containing an additional 20 amino acids in the DNA binding domain (Fig. 2A). It is unclear whether this insertion affects TBX3 + 2a DNA-binding ability or target gene specificity. We therefore compared the capacity of these two TBX3 isoforms to activate *COL1A2* promoter activity in luciferase reporter assays using FLAG-tagged mouse mtbx3 and mtbx3 + 2a expression constructs. Our results show that, albeit to varying degrees, both isoforms were able to activate the *COL1A2* promoter in CT-1 and SW1353 cells (Fig. 2B). Furthermore, a TBX3 mutant (DBM) with a disrupted DNA-binding domain (DBD) and a truncated TBX3 protein lacking the putative activation domain (N-term) were unable to activate the *COL1A2* promoter (Fig. 2C). These data suggest that both the DNA binding and activation domains of the TBX3 protein are required to activate the *COL1A2* promoter in vitro.

Phosphorylation of TBX3 has a direct impact on its protein levels and its ability to bind and regulate target genes [38] and pseudo-phosphorylation of SP190, a highly conserved motif within the DBDs of T-box factors, abolishes the ability of TBX3 to bind and repress the $p21^{WAF1}$ promoter [43]. We therefore tested if this is also true for *COL1A2*. Briefly, CT-1 and SW1353 cells were co-transfected with a *COL1A2* promoter luciferase reporter construct and the pCMV empty vector or expression constructs for wild type (WT) or TBX3 proteins in which the SP190 site was mutated to either alanine (S190A), which abolishes phosphorylation, or glutamic acid (S190E), which mimics phosphorylation. The results show that while WT TBX3 (hTBX3) and S190A TBX3 activate *COL1A2,* pseudo-phosphorylation at S190 abrogated this ability (Fig. 2D). These results suggest that phosphorylation of TBX3 at S190 negatively impacts its activation of *COL1A2*, possibly through physically inhibiting binding of the DBD to the *COL1A2* promoter.

3.2. TBX3 directly binds the COL1A2 promoter

To investigate the region of the *COL1A2* promoter that mediates the activation by TBX3, we next used a luciferase reporter driven by 5' deletion constructs of the *COL1A2* promoter (Fig. 3A). Compared to the



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−2389bp *COL1A2* construct, there was a significant decrease in the ability of TBX3 to activate the −721bp construct and a further decrease in activation of the −375bp *COL1A2* construct. Given that the greatest loss of activation was observed between the −2389bp and −721bp constructs we hypothesised that TBX3 may directly bind the *COL1A2* promoter within this region. Indeed, when chromatin immunoprecipitation assays were performed there was an approximately 15-fold and 2.5-fold enrichment of TBX3 occupancy on the *COL1A2* promoter within this region in CT-1 and SW1353 cells respectively but not on the GAPDH control (Fig. 3B). Manual screening of the region between −2389bp and −721bp identified six putative half T-elements (Fig. 3A). When these sites were disrupted individually

Fig. 3. TBX3 directly binds the *COL1A2* promoter. A. *Upper panel:* Schematic representation of the −2389bp, −721 and −375bp human *COL1A2* promoter luciferase constructs and * indicate the positions of 6 putative T-elements and +1 indicate the transcriptional start site. The forward and reverse primers used for the ChIP assay are indicated by the bold arrows. *Lower panels:* CT-1 transformed fibroblasts and SW1353 chondrosarcoma cells were co-transfected with the indicated *COL1A2* promoter luciferase reporter constructs together with pCMV Empty or hTBX3 expression constructs. The plasmid pCMV Renilla luciferase reporter was used to control for transfection efficiency. Luciferase activity was measured and fold *COL1A2* activation was calculated to that of the Empty pCMV vector transfection data. **B.** CT-1 and SW1353 lysates were used in a ChIP assay with antibodies against TBX3 or IgG (negative control). Immunoprecipitated DNA was subject to qRT-PCR using primers against the *COL1A2* promoter indicated in **A** or GAPDH (negative control). The values indicate the mean of two independent experiments ± SEM (***p < 0.001). **C.** CT-1 and SW1353 cells were co-transfected with the *COL1A2* promoter luciferase reporter constructs in which putative T-elements have been disrupted together with pCMV hTBX3 or the Empty vector. The plasmid pCMV renilla luciferase reporter was used to control for transfection efficiency. Luciferase activity was measured and fold *COL1A2* activation was calculated and normalised to the −2389bp wild type construct. The values indicate the mean of three independent experiments ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001).

using site-directed mutagenesis most of them appear to be capable of mediating activation of the *COL1A2* promoter by TBX3, albeit to different degrees (Fig. 3C). This would suggest that the −2389bp and −721bp region is important for activation of *COL1A2* by TBX3 due to the presence of the many half T-elements. Interestingly, mutating the half T-element at −1258 almost completely abrogated the ability of TBX3 to activate the *COL1A2* promoter. This implies that this site is very important for this activation, and it would be interesting to explore whether TBX3 binds this site. The different effects seen when the various sites were mutated further suggests that some sites may be more important than others. This could perhaps be due to the proximity of these sites to other transcription factor binding sites that may co-regulate the promoter.

3.3. Pseudo-phosphorylation of TBX3 at serine 720 enhances its activation of COL1A2

AKT signalling plays an important role in sarcomagenesis [26–28] and we have previously shown that AKT3 phosphorylation of TBX3 at serine 720 (S720) promotes its protein stability and enhances its ability to repress its target gene, *E-cadherin*, in melanoma cells [38]. We therefore hypothesised that AKT phosphorylation of TBX3 may also impact its protein levels and ability to activate *COL1A2* in fibrosarcoma and chondrosarcoma cells. To explore this, CT-1 and SW1353 cells were treated with the AKTVIII inhibitor and protein from the cells subjected to western blotting with antibodies to TBX3 and COL1A2. Results show that following treatment with the AKT chemical inhibitor there was a decrease in phosphorylated AKT (pAKT) at all time points tested and this corresponded with a significant depletion in endogenous TBX3 and COL1A2 levels (Fig. 4A). Given that AKT exists as three isoforms we next wished to determine which isoform could potentially phosphorylate TBX3 in CT-1 and SW1353 cells. To this end, qRT-PCR analyses were performed to determine the relative mRNA levels of AKT1, AKT2 and AKT3 and the results show that AKT1 is the predominant isoform in both the CT-1 and SW1353 cells (Fig. 4B). To confirm this, we transiently transfected CT-1 and SW1353 cells with an siAKT1 or siCtrl and assessed the levels of TBX3 and COL1A2. The results show that depleting AKT1 caused a substantial decrease in TBX3 with a corresponding decrease in COL1A2 protein (Fig. 4C). Finally, using WT (hTBX3), S720A and S720E TBX3 proteins in luciferase assays we tested if phosphorylation of TBX3 by AKT could impact its ability to transcriptionally activate *COL1A2*. The results reveal that, compared to WT TBX3, the S720E TBX3 activated the *COL1A2* promoter significantly more and the S720A mutant protein had significantly reduced activating ability (Fig. 4D). Western blotting of the lysates assayed showed that equal protein expression was achieved for each of the transfected constructs (lower panel of Fig. 4D). These results suggest that phosphorylation of TBX3 at the AKT motif located at S720 significantly enhances its ability to activate *COL1A2*. Together these data identify a novel link between AKT1, TBX3 and COL1A2 in fibrosarcoma and chondrosarcoma cells.

3.4. COL1A2 mediates the opposing effects of TBX3 on migration of CT-1 transformed fibroblasts and SW1353 chondrosarcoma cells

We next investigated the effect of COL1A2 on the migration of CT-1 and SW1353 cells. To this end, COL1A2 protein levels were transiently knocked down using siRNA and in vitro scratch motility assays were performed. Results show that siCOL1A2 CT-1 cells migrated substantially faster than their siCtrl counterparts (Fig. 5A) whereas a marginal reduction in the migration of siCOL1A2 SW1353 cells was observed (Fig. 5B). Furthermore, western blot analyses show that whereas the knockdown of COL1A2 in CT-1 cells resulted in an increase in N-Cadherin, β-Catenin and Vimentin (Fig. 5A), the depletion of COL1A2 in SW1353 cells caused a decrease in the levels of these molecular markers of migration (Fig. 5B). To determine whether the activation of COL1A2 by TBX3 was responsible for its effects on cell migration, shCtrl and shTBX3 cells were transiently transfected with a *COL1A2* or control expression construct and their migration was assessed. As expected, *COL1A2* overexpression in the CT-1 shCtrl cells resulted in a significant decrease in migration by 12 h (Fig. 6A). Importantly, an even greater decrease in migration was observed in shTBX3 cells overexpressing *COL1A2*, with the rate of migration returning almost to those of the CT-1 shCtrl cells. Conversely, overexpression of *COL1A2* in chondrosarcoma shCtrl cells resulted in a significant increase in migration at 6 and 12-hours (Fig. 6B). Interestingly, overexpression of *COL1A2* in the shTBX3 cells led to increased migratory ability comparable to and greater than that of the SW1353 shCtrl cells at 6 and 12 h respectively. Western blot analyses confirmed the knockdown of endogenous TBX3 in both cell lines and a corresponding decrease in endogenous COL1A2 as well as successful overexpression of ectopic *COL1A2* as indicated (Fig. 6A and B). Together these results show that *COL1A2* is a target of TBX3 and that it is possibly, in part, responsible for mediating the opposite effects of TBX3 on the migration of fibrosarcoma and chondrosarcoma cells.

4. Discussion

TBX3 is overexpressed in a number of carcinomas and sarcomas and an overwhelming body of evidence has implicated it as a key contributor of several oncogenic processes including bypass of apoptosis and senescence as well as promoting proliferation, migration and tumour formation [4,5,9,44–46]. Interestingly, a few studies have suggested that TBX3 may also function as a tumour suppressor. Indeed, we recently reported that although overexpressed in fibrosarcoma, TBX3 inhibits substrate -dependent and -independent cell proliferation, migration and in vivo tumour forming ability [9]. However, the mechanism/s that regulate TBX3's opposing functions remains unknown and there is a paucity of information regarding its target genes. Here we demonstrate that COL1A2 is a novel target activated by TBX3 in fibrosarcoma and chondrosarcoma and that while COL1A2 functions downstream of TBX3 to inhibit fibrosarcoma cell migration, it promotes chondrosarcoma cell migration.

TBX3 is a well-known transcriptional repressor but there are some reports that have identified and characterised genes that are activated by TBX3 during early embryonic development [47–49]. For example,

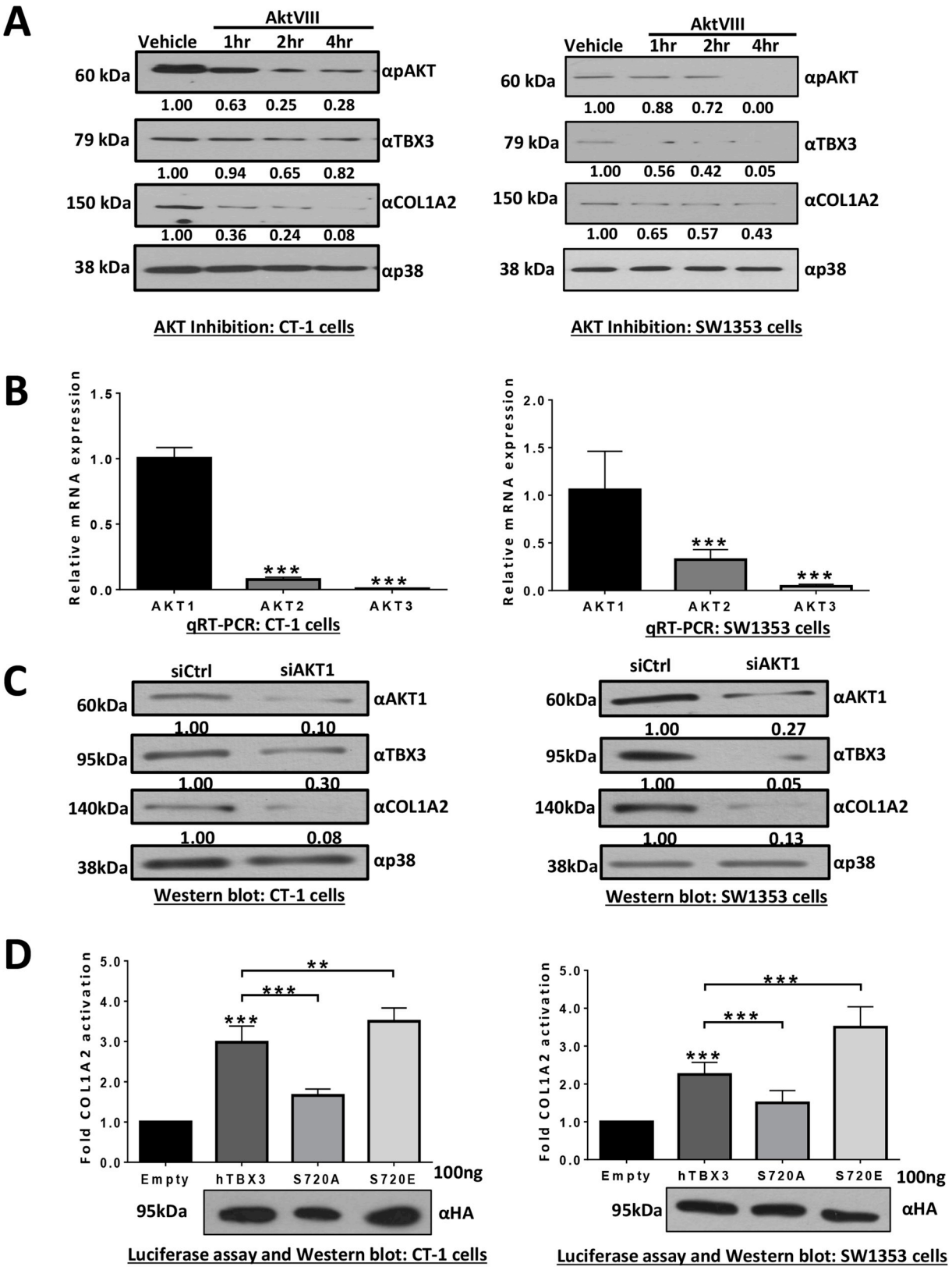

Fig. 4. Pseudo-phosphorylation of TBX3 at serine 720 enhances its activation of *COL1A2* promoter. **A.** CT-1 transformed fibroblasts and SW1353 chondrosarcoma cells were serum starved for 24 h followed by treatment with vehicle (DMSO) or 10 μM AKTVIII for 1, 2 or 4 h after which protein extracts were harvested and analysed by western blotting using the indicated antibodies. Total p38 was used as a loading control. Densitometric analysis was performed relative to p38. **B.** CT-1 and SW1353 cDNA was subjected to qRT-PCR using primers specific to AKT1, AKT2 and AKT3 and normalised to GusB. **C.** Western blot analysis of CT-1 and SW1353 cells transiently transfected with 10 nM siAKT1 or siCtrl and antibodies to AKT1, TBX3 and COL1A2. Densitometry was calculated relative to the p38 loading control. **D.** CT-1 and SW1353 cells were co-transfected with the *COL1A2* promoter luciferase reporter together with HA-tagged pCMV Empty, WT hTBX3, hTBX3 S720A or hTBX3 S720E expression vectors. The pCMV renilla luciferase reporter was used to control for transfection efficiency. Luciferase activity was measured and fold activation of the *COL1A2* promoter was calculated and normalised to that of the empty pCMV vector transfection. *Lower panels*: Western blots of transfected TBX3 proteins detected using an antibody to HA. **B, D.** The values indicate the mean of three independent experiments ± SEM (*p < 0.05; **p < 0.01;

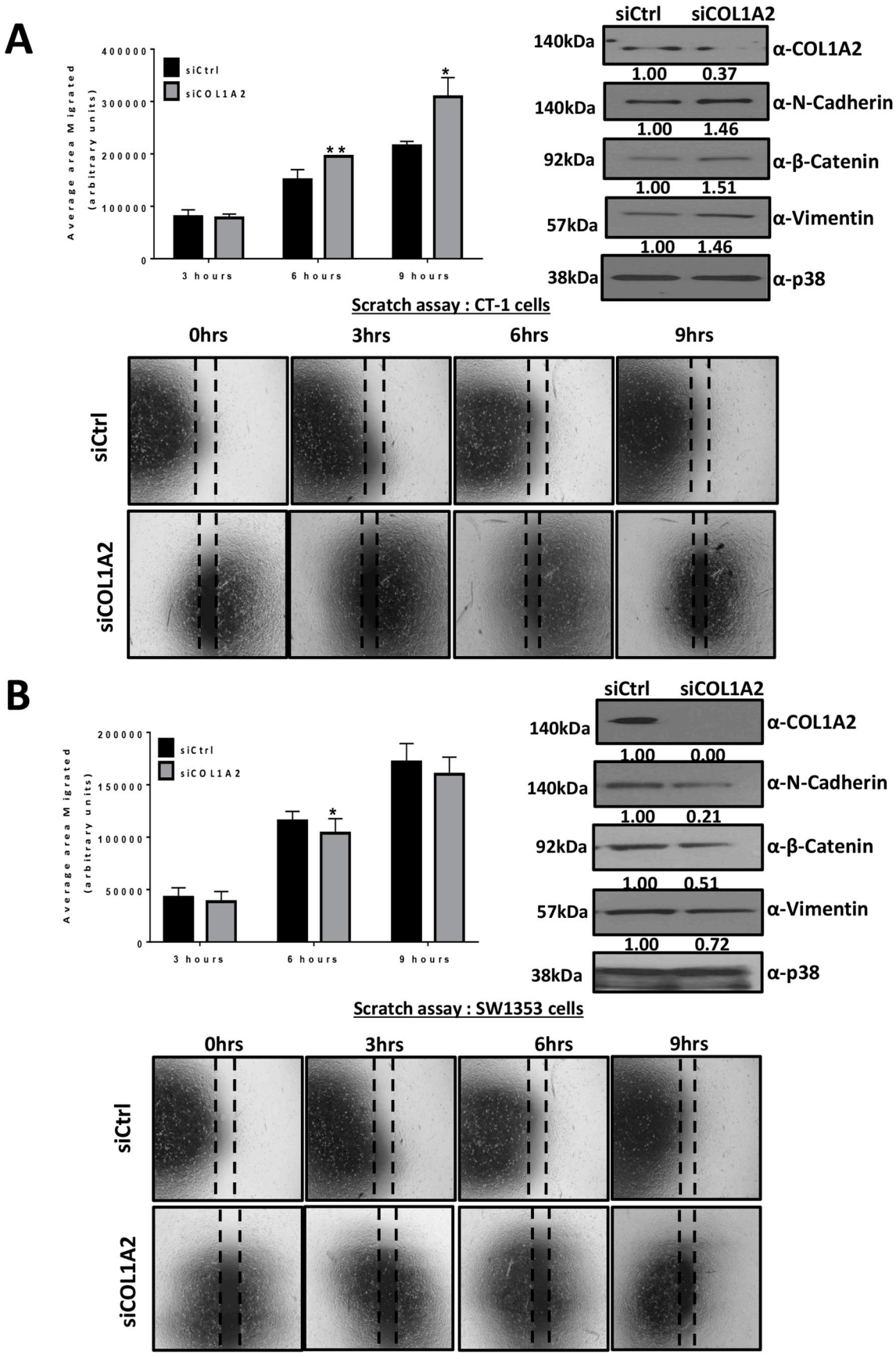

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Fig. 5. COL1A2 inhibits fibrosarcoma cell migration but promotes chondrosarcoma cell migration. A and B. CT-1 transformed fibroblasts and SW1353 chondrosarcoma cells were transiently transfected with 5 nM siCOL1A2 or siCtrl for 48 h, a linear wound was made on confluent cells and histograms show the distance migrated at 3, 6 and 9 h. The values indicate the mean of three independent experiments ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001). *Lower panels:* photographs of the linear wounds at 0, 3, 6 and 9 h. Western blots on the *right* confirm successful knockdown of COL1A2 as well as the expression of the molecular markers of migration, N-Cadherin, β-Catenin and Vimentin. Densitometry was calculated relative to the p38 loading control.

Weidgang et al. (2013) showed that in mouse embryonic stem cells (mESCs), TBX3 directly bound and activated the promoters of *Eomes, T* and *Sox17* which are essential for mesoderm differentiation [50]. Lu et al. (2011) also reported that TBX3 directly binds and activates the *Gata6* promoter in mESCs to promote extra embryonic endodermal differentiation [51]. In the cancer context, however, there is only evidence of TBX3 functioning as a transcriptional repressor and the current study is therefore the first to show that TBX3 is capable of activating a target gene to impact oncogenic transformation. Indeed, TBX3 directly represses transcription of the tumour suppressors, $p19^{ARF}$ ($p14^{ARF}$ in humans) in primary breast tumours [1], cyclin-dependent kinase inhibitor $p21^{WAF1}$ in chondrosarcoma [2], *pTEN* in head and neck squamous carcinoma [3] and *E-cadherin* in melanoma [4]. Full characterisation of the activation of the *COL1A2* promoter by TBX3 reveals that the DNA binding domain as well as the region of the TBX3 protein containing the activation domain are required for this activity. The importance of the DNA binding and activation domains for the function of TBX3 is consistent with studies that showed that in UMS sufferers, 50% of TBX3 mutations occur within the DNA binding domain while the remaining 50% were mapped to the C-terminus which harbours the activation domain [1].

Based on published reports, the mechanism by which TBX3 regulates its target genes appears to involve single half T-elements. For example, TBX3 binds and represses $p14^{ARF}$ through CACCTCTGGTG CCA, *E cadherin* through CAGGTGT and $p21^{WAF1}$ through GTGTGA close to the initiator [43,52,53]. Interestingly, we identified 6 putative half T-elements in the *COL1A2* promoter and using site-directed mutagenesis and luciferase assays we observed that disruption of at least 4 of these resulted in a significant reduction in the ability of TBX3 to activate *COL1A2,* albeit it to varying degrees. This suggests that TBX3 may be activating *COL1A2* through more than 1 single half T-element and that some sites may be more important than others. Additional DNA binding assays to investigate which of the putative half T-elements in the *COL1A2* promoter are directly bound by TBX3, especially those that appeared to be important following site-directed mutagenesis as shown in Fig. 3C, should be the focus of future studies. Interestingly, the T-box transcription factor T-bet is also able to recognise and bind multiple T half sites and may preferentially bind sequences with certain core motifs arranged in various orientations [54]. This may account for the varying degrees in which mutations of the individual half T-elements affect TBX3's ability to activate the *COL1A2* promoter. It is also possible that the proximity of the half T-elements to other transcription factor binding sites may co-regulate the promoter. Indeed, it is possible that cooperative promoter activation may result from TBX3 occupancy at certain T-half sites together with other transcriptional regulators which create a strong DNA-TBOX-regulator complex similar to that reported by Bruneau et al. (2001) for the Tbx5/Nkx2-5 complex on *Nppa* during murine cardiac development [55].

Post-translational modifications, including phosphorylation, of transcription factors play an important role in the regulation of gene expression in eukaryotes because it can impact transcription factor target gene binding and regulation [56]. Serine-proline motifs are consensus sites for a number of kinases including KIT, MAPK, PDGFRA and AKT [57,58] and there are 11 SP motifs in the TBX3 protein. Of these, SP190 occurs in the T-box (DNA binding domain) and is highly conserved across several species and among other T-box factors. It is therefore anticipated that the transcriptional activity of TBX3, as well as other T-box factors, will be regulated by phosphorylation at this site. Here we show that pseudo-phosphorylation of SP190 significantly abrogated the ability of TBX3 to activate the *COL1A2* promoter. This is in line with a previous study that showed that pseudo-phosphorylation at SP190 also abolished the ability TBX3 to bind and repress the $p21^{WAF1}$ promoter [43]. Future studies should attempt to identify the kinase(s) that phosphorylate TBX3 at SP190 because this will provide an additional mechanism by which the oncogenic activity of TBX3 can potentially be inhibited. This approach would be beneficial as several protein kinases are thought to function as tumour suppressors because they are often lost or inhibited in different cancers due to loss-of-function mutations [59]. Indeed, the loss of PKCδ activity was recently shown to promote colon cancer and a recent study by Bessa et al. (2018) investigated the possibility of re-activating this kinase as a potential treatment strategy for colon cancer [60–65]. The authors demonstrate that a selective small molecule activator of PKCδ (Roy-Bz) in colon cancer induced a PKCδ-dependent anti-tumour effect through anti-proliferative, pro-apoptotic, and anti-angiogenic activities.

Activation of the PI3K/AKT signalling pathway has well established tumour promoting roles in many human cancers [66–70]. Indeed, AKT phosphorylates substrates that promote tumour cell proliferation, invasion and metastasis and there is evidence that TBX3 may be one such substrate [71,72]. TBX3 has one AKT consensus motive at S720 and in melanoma, AKT3 phosphorylates TBX3 at this site which results in an increase in TBX3 stability as well as its ability to transcriptionally repress E-cadherin to promote migration [45]. Interestingly, here we show that AKT1 is the dominant isoform in the fibrosarcoma and chondrosarcoma cells tested and that it is a key upstream regulator of TBX3 in these two sarcoma subtypes. Indeed, we show that TBX3 levels are positively regulated by AKT1 in fibrosarcoma and chondrosarcoma and that pseudo-phosphorylation of TBX3 at S720 significantly increased its ability to activate the COL1A2 promoter in both sarcomas. This suggests that despite the overwhelming evidence to support tumour promoting roles for AKT signalling, AKT1 may function as either tumour promoter or tumour suppressor. Indeed, albeit only one report, there is some indication that AKT1 and 2 may have tumour suppressor roles [73]. The authors show that Akt2 (−/−) mice survived hepatic Akt1 deletion, but all developed spontaneous hepatocellular carcinoma [73]. Our data therefore contributes to a new body of information that suggests that the PI3K/AKT signalling pathway can either drive or suppress cancer progression depending on cellular context. These findings are important because they suggest that the use of AKT inhibitors for the treatment of cancer need to be carefully considered.

Our study revealed that whereas COL1A2 inhibited fibrosarcoma cell migration, it promoted chondrosarcoma cell migration. These results are consistent with studies that have demonstrated that COL1A2 may have tumour suppressor roles in certain malignancies and tumour promoting roles in other malignancies [19,74–78]. We speculate that the ability of COL1A2 to function as either tumour promoter or suppressor may be due to it interacting with different protein partners in different contexts which may in turn impact unique downstream target molecules of COL1A2. Indeed, this possibility is supported by a recent published study which showed a strong interaction between COL1A2 and MMP9 and the promotion of colon cancer. In the study, the authors analysed microarray datasets which compared colon tumours with their adjacent normal mucosa and constructed a protein-protein interaction network of differentially-expressed genes [79]. MMP9 degrades various components of the ECM, including type I collagen, and numerous studies have shown that it plays crucial roles in cell proliferation, angiogenesis, invasion and metastasis [79–82]. It would be important to determine whether COL1A2 interacts with MMP9 in chondrosarcoma

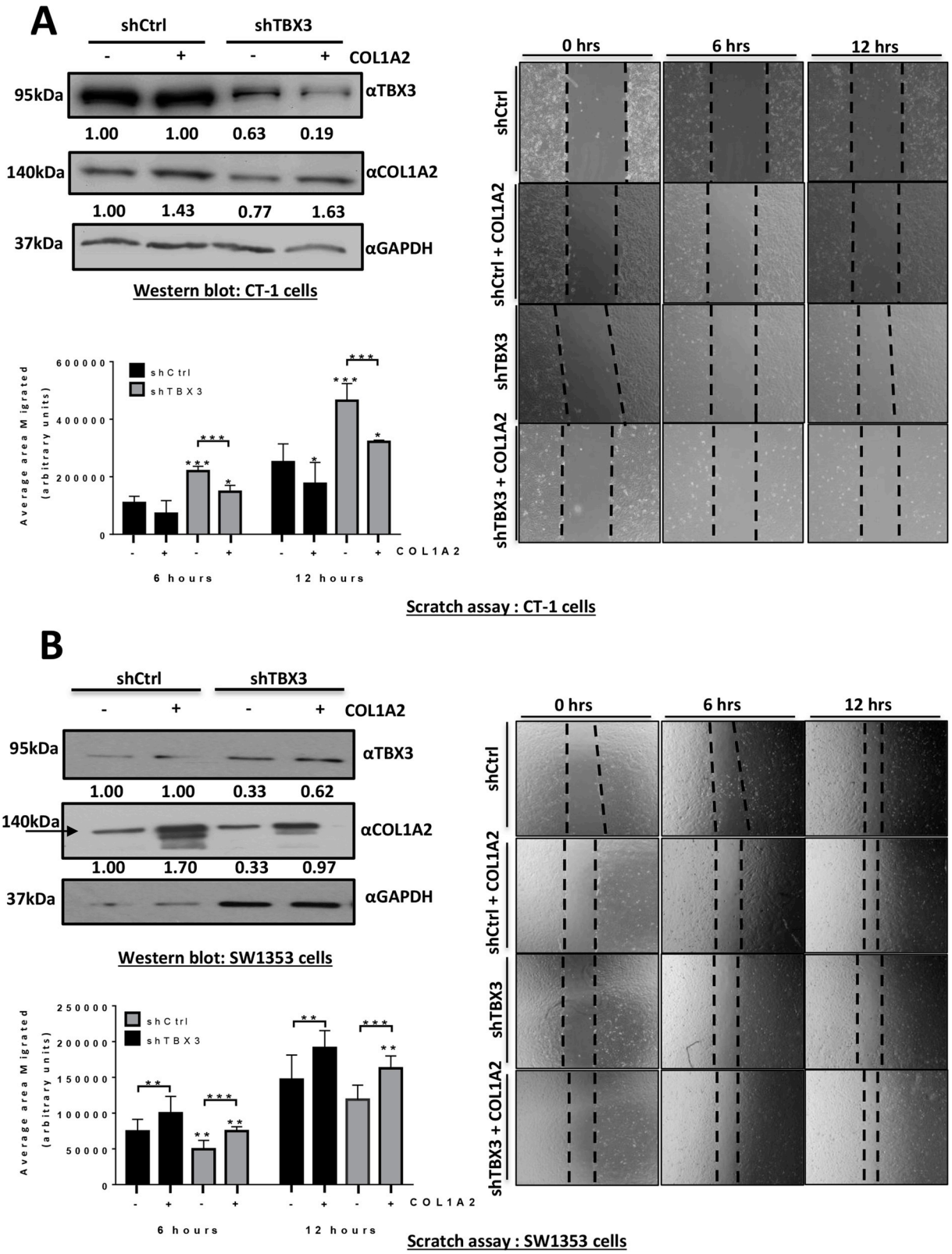

Fig. 6. COL1A2 mediates the opposing effects of TBX3 on migration of CT-1 transformed fibroblasts and SW1353 chondrosarcoma cells. A and B. CT-1 and SW1353 cell lines in which TBX3 was stably knocked down (shTBX3) or their control (shCtrl) were transiently transfected with (+) or without (−) the human COL1A2 expression construct. A linear wound was made on confluent CT-1 and SW1353 cells and the histograms show distance migrated at 6 and 12 h. The values indicate the mean of three independent experiments ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). *Right panels:* photographs of the linear wounds taken at 0, 6 and 12 h. Western blot analyses confirm expression of indicated proteins. GAPDH was used as a loading control and densitometry shows expression normalised to GAPDH.

cells but not fibrosarcoma cells because this may reveal the mechanism by which COL1A2 promotes migration in the one case but not the other. It would also be interesting to compare COL1A2-regulated genes in fibrosarcoma and chondrosarcoma cells because this could shed further light on the opposite effects of COL1A2 on migration of these two sarcoma subtypes. Finally, it will be useful for these studies to include more than one cell line representative of fibrosarcoma and chondrosarcoma.

In conclusion, our data reveal a novel AKT1/TBX3/COL1A2 axis that may function to either promote or inhibit sarcomagenesis. These findings have important implications for the design of therapeutic strategies in cancers driven by this axis.

Conflicts of interest

None.

Acknowledgements

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