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A novel role for the anti-senescence factor TBX2 in DNA repair and cisplatin resistance

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The emergence of drug resistant tumours that are able to escape cell death pose a major problem in the treatment of cancers. Tumours develop resistance to DNA-damaging chemotherapeutic agents by acquiring the ability to repair their DNA. Combination therapies that induce DNA damage and disrupt the DNA damage repair process may therefore prove to be more effective against such tumours. The developmentally important transcription factor TBX2 has been suggested as a novel anticancer drug target, as it is overexpressed in several cancers and possesses strong anti-senescence and pro-proliferative functions. Importantly, we recently showed that when TBX2 is silenced, we are able to reverse several features of transformation in both breast cancer and melanoma cells. Overexpression of TBX2 has also been linked to drug resistance and we have shown that its ectopic expression results in genetically unstable polyploidy cells with resistance to cisplatin. Whether the overexpression of endogenous TBX2 levels is associated with cisplatin resistance in TBX2-driven cancers has, however, not been shown. To address this we have silenced TBX2 in a cisplatin-resistant breast cancer cell line and we show that knocking down TBX2 sensitises the cells to cisplatin by disrupting the ATM-CHK2-p53 signalling pathway. Cell cycle analyses demonstrate that when TBX2 is knocked down there is an abrogation of an S-phase arrest but a robust G2/M arrest that correlates with a reduction in phosphorylated CHK2 and p53 levels. This prevents DNA repair resulting in TBX2-deficient cells entering mitosis with damaged DNA and consequently undergoing mitotic catastrophe. These results suggest that targeting TBX2 in combination with chemotherapeutic drugs such as cisplatin could improve the efficacy of current anticancer treatments.
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Malignant neoplasm is the second leading cause of death world-wide with breast cancer being the most common in women.[1] Currently, the prevalent subtypes of breast cancer are treated using a targeted approach involving antioestrogens, antiprogesterones or inhibitors of the human epithelial growth factor receptor 2.[2,3] One major problem is, however, the recurrence of drug resistant tumours[2] and the treatment of triple negative breast cancers.[4] Although the use of platinum-based chemotherapy such as cisplatin has shown promising results in the treatment of these breast cancers, it has become evident that combination therapies will increase their efficacy.[5–7]

Cisplatin is a widely used chemotherapeutic drug that triggers apoptosis through inducing DNA damage.[6] There is, however, evidence to suggest that tumours develop cisplatin resistance in part through the ability to repair drug-induced DNA damage and consequently evading apoptosis.[8,9] Combining cisplatin with a targeted approach that involves dismantling the DNA damage repair process may overcome this problem. The ATM-CHK2-p53 signalling cascade has an important role in repairing cisplatin-induced DNA damage. Once DNA damage is detected, activated ATM and its downstream target CHK2 phosphorylate p53 that controls cell fate by either initiating cell cycle arrest to allow for DNA repair or by triggering cell death pathways if the DNA damage is too severe.[9–11]

The developmentally important transcription factor TBX2 overexpressed in several cancers including a subset of aggressive breast cancers.[12] Accumulating evidence suggests that this overexpression results in the deregulation of key cell cycle checkpoints to promote proliferation.[13–19] For example, TBX2 is able to bypass senescence through its ability to repress the cell cycle regulators p21 and ARF[14,18] and silencing TBX2 induces senescence in melanoma cells.[13,17] Furthermore, TBX2 was shown to promote proliferation of the MCF-7 breast cancer cells through co-operating with EGR1 to repress NDRG1, and knocking down TBX2 in these cells strongly inhibited their proliferation.[13,16] Interestingly, the ectopic expression of TBX2 has also been linked to conferring resistance to the DNA-damaging chemotherapeutic drugs, cisplatin and doxorubicin.[20,21] Whether the overexpression of endogenous TBX2 levels is associated with cisplatin resistance in TBX2-driven cancers has, however, not been shown.

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Abbreviations: ARF, alternative reading frame (p19); ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia and Rad3 related; BRCA 1, breast cancer 1; BRCA 2, breast cancer 2; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; EGR1, early growth response 1; G1, gap phase 1; G2/M, gap phase 2/Mitosis; GUSB, glucuronidase beta; KSP/Eg5, kinesin spindle protein Eg5; NDRG1, N-myc downregulated gene 1; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; qRT-PCR, quantitative real time PCR; RPMI, Roswell Park Memorial Institute; RT, room temperature; S-phase, synthesis phase; siRNA, small interfering RNA; TBX2, T-box 2; H2AX, H2A histone family, member X
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Here we show that depleting TBX2 in combination with cisplatin treatment leads to cell death and drug sensitisation in cisplatin-resistant MCF-7 cells. We demonstrate that this occurs through disruption of the ATM-CHK2 signalling pathway and destabilised p53. This correlates with an impaired S-phase arrest that does not allow for DNA repair and consequently cells undergo mitotic catastrophe. These results support evidence that TBX2 is a potential novel drug target that can be used in combination therapies with DNA-damaging chemotherapeutic agents to improve their efficacy.

Results

Increased sensitivity of MCF-7 and 501mel cells to cisplatin after TBX2 knockdown. On the basis of previous observations, we hypothesised that knocking down TBX2 in TBX2-driven cancers may sensitise these cells to cisplatin treatment.[13,20] To explore this possibility, MCF-7 breast cancer cells were used as a system because they overexpress TBX2[14] and are reported to be cisplatin resistant.[22,23] MCF-7 cells in which TBX2 was knocked down by RNA interference (shTBX2) and their control cells (shCtrl) (Figure 1a) were treated with cisplatin and cell viability assessed using clonogenic survival assays. Figure 1b shows that, as previously reported, knocking down endogenous TBX2 reduced the proliferation of untreated cells[13,16] but importantly it resulted in chemosensitivity to cisplatin. A similar trend could be confirmed in the previously described shCtrl- and shTBX2-501 melanoma cells (501mel; Peres *et al*.[13]) (Figures 1c and d). Compared with the MCF-7

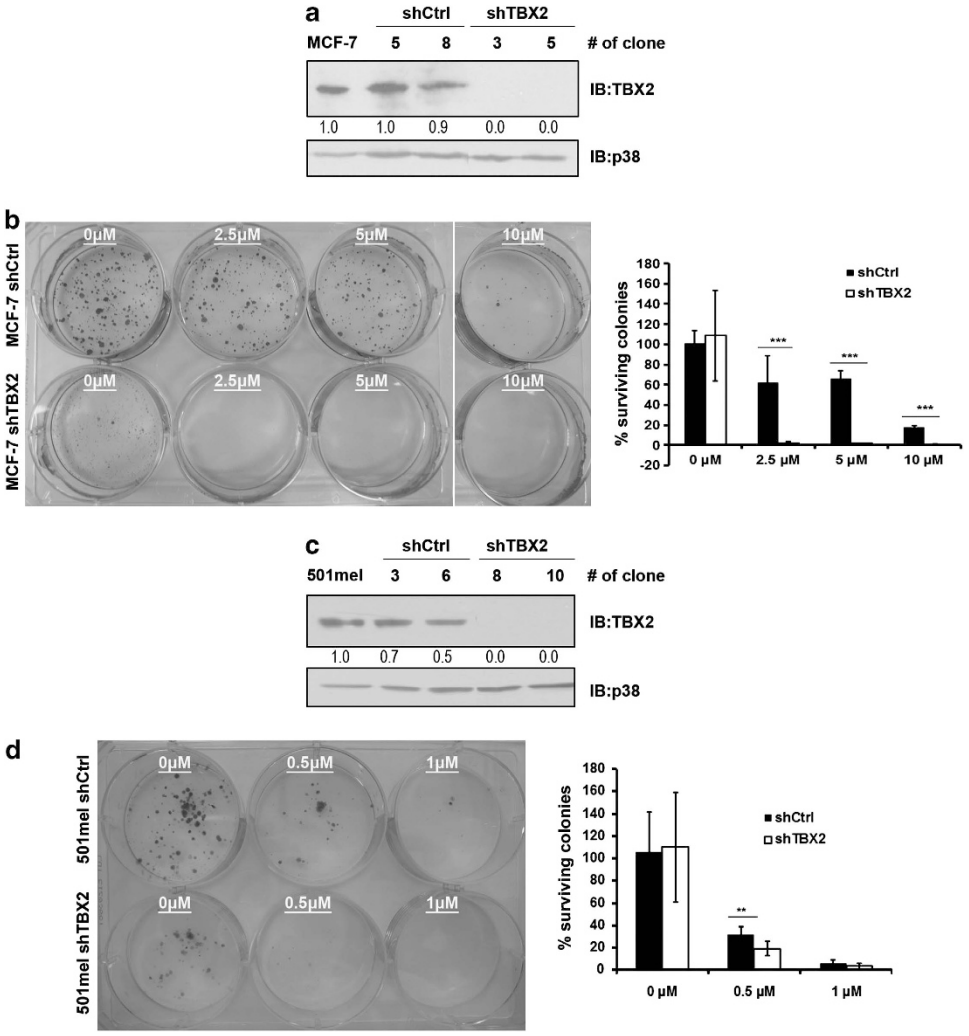
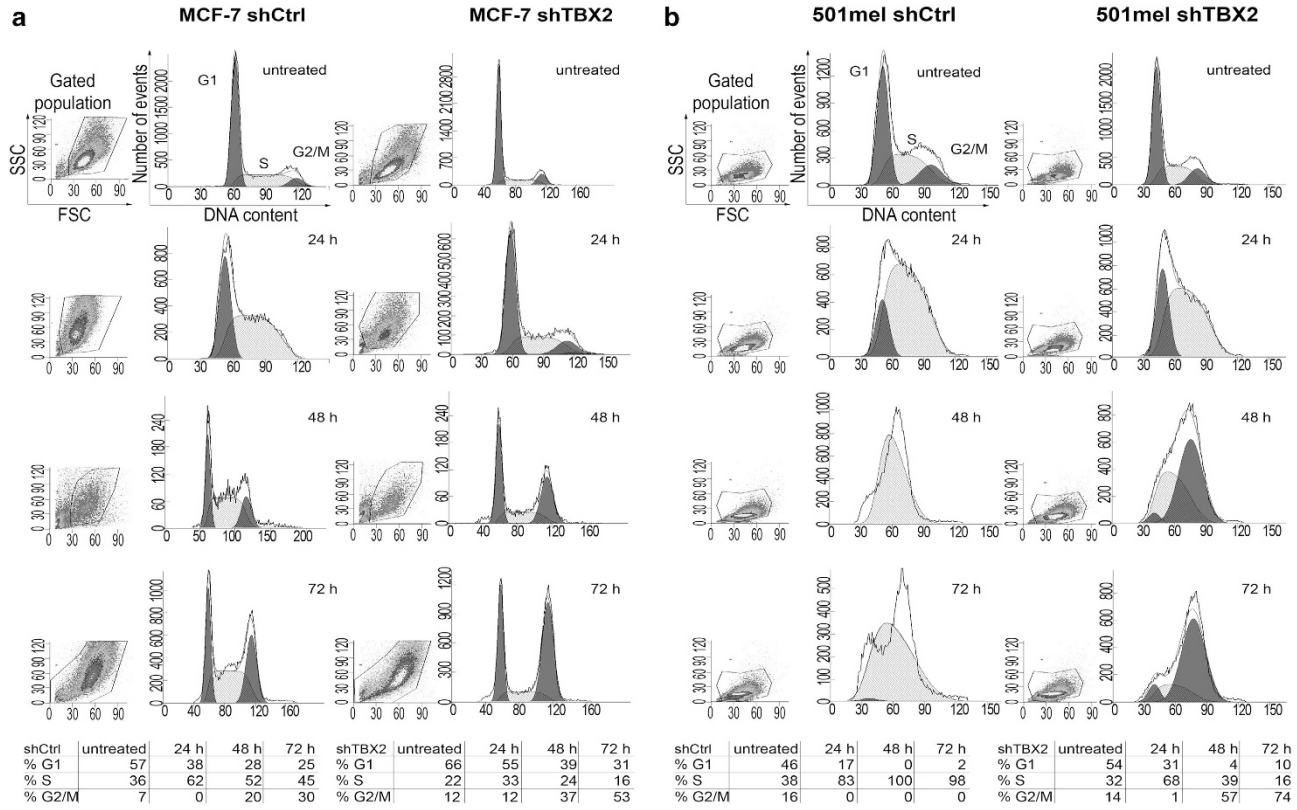


Figure 1 Silencing TBX2 sensitises MCF-7 and 501mel cells to cisplatin. (**a**) Western blot showing TBX2 protein levels of parental MCF-7 cells and in MCF-7 shCtrl and MCF-7 shTBX2 clones. Total p38 protein levels were used as a loading control. The expression of TBX2 was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**b**) MCF-7 shCtrl and shTBX2 cells were treated with 0, 2.5, 5 and 10 μM cisplatin for 24 h, washed, replated at low density and incubated for 28 days. The results of three replicates were analyses and show the average percentage of surviving colonies. ***P-value < 0.001. (**c**) Western blot showing TBX2 protein levels of parental 501mel cells and in 501mel shCtrl and shTBX2 clones. Total p38 protein levels were used as a loading control. The expression of TBX2 was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**d**) 501mel shCtrl and shTBX2 cells were treated with 0, 0.5 and 1 μM cisplatin for 24 h, washed, replated at low density and incubated for 14 days. The results of three replicates were analyses and show the average percentage of surviving colonies. **P-value < 0.01

cells, however, the 501mel cells were more sensitive to cisplatin as indicated by the concentration of cisplatin used in the assay.

S-phase arrest induced by cisplatin requires TBX2 expression in MCF-7 and 501mel cells. Cisplatin resistance is thought to arise from cancer cells preferentially arresting in S-phase to repair the DNA damage induced by the drug.[8,9] The above results suggested that the overexpression of TBX2 in MCF-7 cells may contribute to conferring cisplatin resistance. To begin to explore this, shCtrl and shTBX2 cells were incubated with cisplatin for 24, 48 and 72 h and their cell cycle distribution analysed by flow cytometry. Consistent with the published literature,[24,25] a large proportion of the shCtrl cells were arrested in S-phase especially at 24 h (Figure 2a). In contrast, only a small population of shTBX2 cells were arrested in S-phase at 24 h and, compared with shCtrl cells, many more cells arrested in G2/M at 48 and 72 h. Figure 2b shows that these results could be confirmed in the shCtrl- and shTBX2-501mel cells.[13] However, the 501mel shCtrl cells maintained an S-phase arrest for the duration of the treatment, and the 501mel shTBX2 cells did arrest in the S-phase at 24 h but this arrest was not maintained and again the majority of the cells were arrested in G2/M at 48 and 72 h. Taken together, the above data showed that although the MCF-7 and 501mel control cells preferentially arrested in S-phase in response to cisplatin treatment, the absence of TBX2 appeared to compromise this ability and favoured a G2/M arrest.

Targeting TBX2 in combination with cisplatin treatment induces mitotic catastrophe. There is evidence to suggest that an S-phase arrest promotes chemotherapeutic drug resistance because it enables DNA damage repair and hence cell survival.[9,26–28] We therefore hypothesised that the weakened S-phase arrest observed for the cisplatin-treated shTBX2 cells prevents DNA repair. To test this we subjected cisplatin-treated MCF-7 shCtrl and shTBX2 cells to immunocytochemistry with an antibody to the DNA damage marker γ-H2AX. Indeed, quantitative analyses of the data showed that, compared with shCtrl cells, significantly more shTBX2 cells express γ-H2AX, which was particularly striking at 72 h (Figure 3a). These results were confirmed by western blotting, which showed that although an increase in γ-H2AX was observed in shCtrl cells at 24 and 48 h of cisplatin treatment, it was undetectable at 72 h (Figure 3b). These results together with the cell cycle analyses in Figure 2a suggest that compared with shCtrl cells, shTBX2 cells enter mitosis with damaged DNA and may therefore undergo mitotic catastrophe.[29] The next set of experiments therefore examined cisplatin-treated MCF-7 shCtrl and shTBX2 cells for the coexpression of γ-H2AX and the mitotic marker cyclin B1. Figure 3b shows that unlike the shCtrl cells, the shTBX2



shCtrl	untreated	24 h	48 h	72 h
% G1	57	38	28	25
% S	36	62	52	45
% G2/M	7	0	20	30

shTBX2	untreated	24 h	48 h	72 h
% G1	66	55	39	31
% S	22	33	24	16
% G2/M	12	12	37	53

shCtrl	untreated	24 h	48 h	72 h
% G1	46	17	0	2
% S	38	83	100	98
% G2/M	16	0	0	0

shTBX2	untreated	24 h	48 h	72 h
% G1	54	31	4	10
% S	32	68	39	16
% G2/M	14	1	57	74

Figure 2 Silencing TBX2 results in an abrogation of a cisplatin-induced S-phase arrest in MCF-7 and 501mel cells. (**a**) MCF-7 shCtrl and MCF-7 shTBX2 cells were treated with 10 μM cisplatin for 24, 48 and 72 h and cell cycle profiles analysed by flow cytometry. (**b**) 501mel shCtrl and 501mel shTBX2 cells were treated with 5 μM cisplatin for 24, 48 and 72 h and cell cycle profiles analysed by flow cytometry. FSC, forward scatter; SSC, side scatter

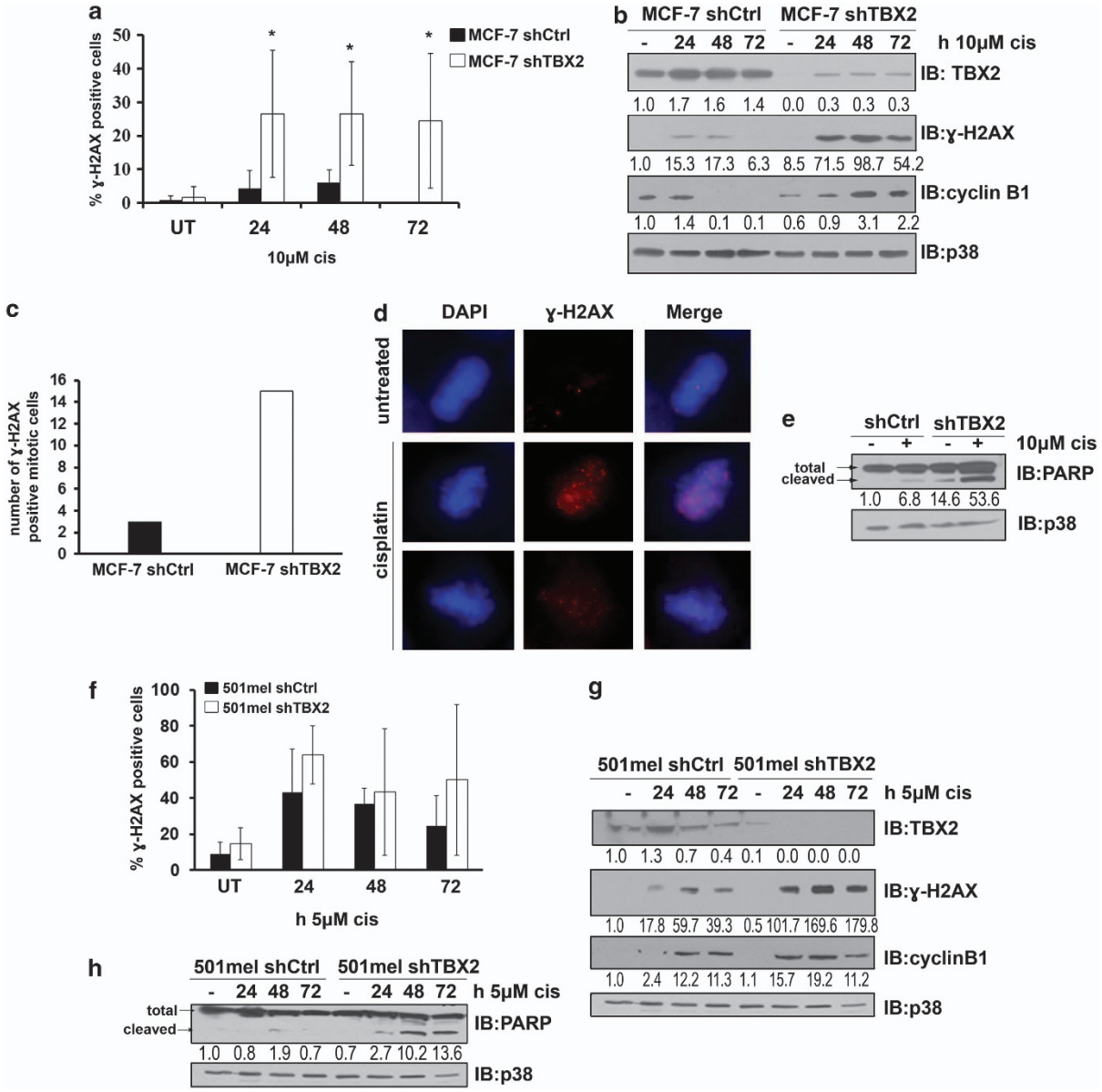


Figure 3 Cisplatin-treated shTBX2 cells undergo mitotic catastrophe. (**a**) MCF-7 shCtrl and shTBX2 cells were treated with 10 μM cisplatin for 24, 48 and 72 h and analysed by immunocytochemistry with an antibody to γ-H2AX and visualised by confocal microscopy. The average percentage of γ-H2AX-positive cells obtained from five random fields of view is represented. Error bars represent S.D. **P*-value < 0.05. (**b**) MCF-7 shCtrl and MCF-7 shTBX2 cells were treated as described in (**a**), and TBX2, γ-H2AX and cyclin B1 protein levels were assessed by western blotting. Total p38 protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**c**) Cells were treated with 10 μM cisplatin for 72 h and analysed by immunocytochemistry with an antibody to γ-H2AX and visualised by fluorescent microscopy ($\times$ 100). A comparison of γ-H2AX-positive mitotic cells detected in MCF-7 shCtrl *versus* MCF-7 shTBX2 cells is shown. (**d**) Representative examples of untreated and cisplatin-treated MCF-7 shTBX2 cells. (**e**) MCF-7 shCtrl and shTBX2 cells were treated with 10 μM cisplatin for 120 h, and the protein levels of total and cleaved PARP were determined by western blot analysis. Total p38 protein levels were used as a loading control. The expression of cleaved PARP was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**f**) 501mel shCtrl and 501mel shTBX2 cells were treated with 5 μM cisplatin for 24, 48 and 72 h and analysed by immunocytochemistry with an antibody to γ-H2AX and visualised by confocal microscopy. The average percentage of γ-H2AX-positive cells obtained from five random fields of view is represented. Error bars represent S.D. (**g**) 501mel shCtrl and 501mel shTBX2 cells were treated as described in (**f**) and TBX2, γ-H2AX and cyclin B1 protein levels were assessed by western blotting. Total p38 protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**h**) 501mel shCtrl and 501mel shTBX2 cells were treated as described in (**f**) and protein levels of total and cleaved PARP were determined by western blot analysis. Total p38 protein levels were used as a loading control. The expression of cleaved PARP was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels

cells did indeed undergo mitotic catastrophe because they have elevated levels of cyclin B1 with correspondingly high levels of γ-H2AX at all the time points tested. These results were confirmed by immunocytochemistry with an antibody to γ-H2AX and DAPI staining that shows DNA damage and the characteristic condensed chromatin of mitotic cells, respectively. Cells were treated with cisplatin for 72 h and the whole population of cells was scanned for the presence of mitotic

cells. Our analyses revealed that when the same number of mitotic cells in each cell line was compared, there were five times more γ-H2AX-positive mitotic cells in the shTBX2 cells (Figure 3c). Examples of untreated and treated shTBX2 cells are shown in Figure 3d.

To determine whether the mitotic catastrophe seen in shTBX2 cells treated with cisplatin lead to apoptosis, we next assessed the effect of knocking down TBX2 on PARP cleavage. The results showed that the levels of both total and cleaved PARP are indeed higher in the shTBX2 cells compared with the shCtrl cells at 120 h of cisplatin treatment (Figure 3e). It is worth noting that untreated shTBX2 cells also have higher levels of both total and cleaved PARP compared with untreated control cells. This would suggest that knocking down TBX2 alone activates the apoptotic pathways. When the above experiments were repeated in the 501mel cells, similar results were obtained (Figures 3f–h) and together with the cell cycle analyses in Figure 2b suggest that 501mel shTBX2 cells also enter mitosis with damaged DNA and undergo cell death. Taken together, these data suggest that TBX2 has a role in determining the response of TBX2-expressing cancer cells to cisplatin treatment and that depleting TBX2 levels in these cells prevents DNA damage repair in S-phase and promotes cell death.

TBX2 is required for intact ATM-CHK2-p53 signalling following cisplatin treatment. To explore a possible role for TBX2 in the DNA damage response, we first examined the signalling pathways known to trigger the S-phase arrest.[30,31] To this end, the shCtrl and shTBX2 cells were treated with cisplatin as previously described and the ATM-CHK2-p53-mediated DNA damage response was assessed (Figure 4a). As judged by the levels of phosphorylated ATM (p-ATM; Ser1981) it would appear that both shCtrl and shTBX2 cells were able to detect the DNA damage induced by cisplatin (Figures 4b and c). The shCtrl cells, however, showed a robust activation of CHK2 (Thr68; p-CHK2), whereas cells depleted of TBX2 had a negligible CHK2 response, which suggest that TBX2 is positioned downstream of ATM but upstream of CHK2 (Figures 4b and c).

When activated, both ATM and CHK2 can phosphorylate p53 resulting in the stabilisation and activation of this tumour suppressor, which determines cell fate by either initiating DNA repair and survival or cell death. Furthermore, the MCF-7 cells are reported to express wild-type p53, which in response to DNA damage is activated by ATM and CHK2[22,32] and thus the next obvious question that arose was whether the p53 response was intact in the MCF-7 shCtrl cells but impaired in the shTBX2 cells. Western blot analyses showed that, whereas the shCtrl cells exhibited a robust increase in the p53 levels after cisplatin treatment, the shTBX2 cells expressed undetectable or lower levels of p53 protein during the time points investigated (Figure 4d). The cyclin-dependent kinase inhibitor p21 is a p53 target gene and has an important role in initiating cell cycle arrests following DNA damage.[8,33] It was therefore hypothesised that active p53 in cisplatin-treated shCtrl cells would lead to the upregulation of p21, which would be impaired in shTBX2 cells and this is indeed shown in

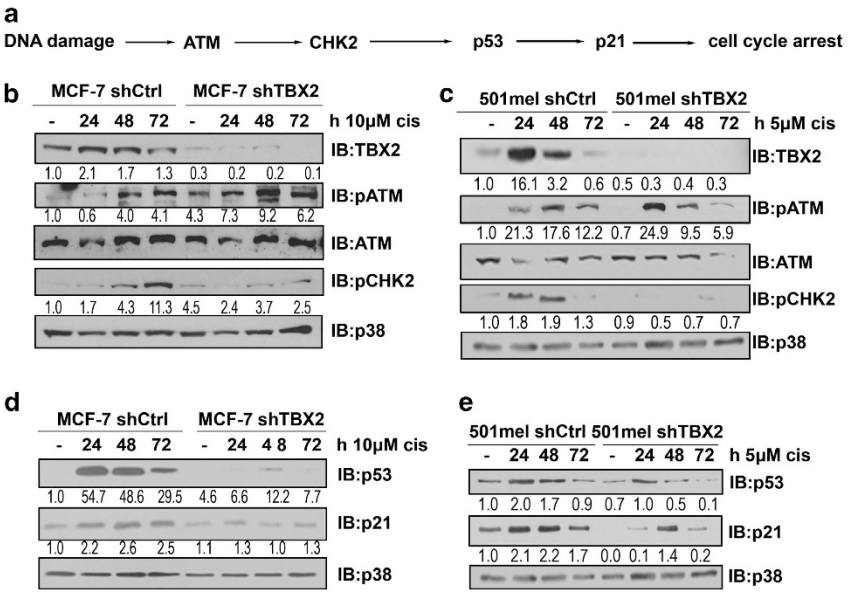
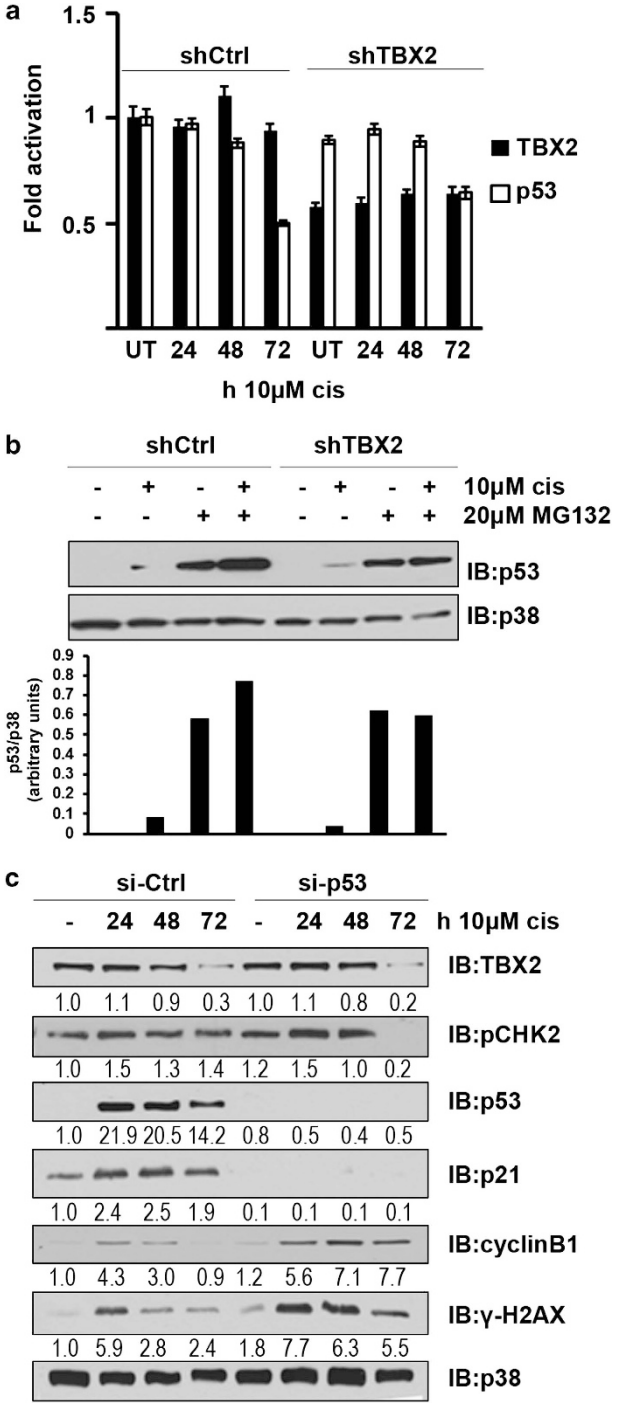


Figure 4 Cells lacking TBX2 show impaired ATM-CHK2-p53 signalling after cisplatin treatment. (**a**) Schematic diagram of the ATM DNA damage signalling cascade. (**b**) MCF-7 shCtrl and MCF-7 shTBX2 cells were treated with 10 μM cisplatin for 24, 48 and 72 h, and TBX2, p-ATM (Ser1981), ATM, p-CHK2 (Thr68) protein levels were assessed by western blotting. Total p38 and ATM protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 or ATM levels. (**c**) 501mel shCtrl and 501mel shTBX2 cells were treated with 5 μM cisplatin for 24, 48 and 72 h, and TBX2, p-ATM (Ser1981), ATM, p-CHK2 (Thr68) protein levels were assessed by western blotting. Total p38 and ATM protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 or ATM levels. (**d**) MCF-7 shCtrl and MCF-7 shTBX2 cells were treated as in (**b**), and p53 and p21 protein levels were assessed by western blotting. Total p38 protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**e**) 501mel shCtrl and 501mel shTBX2 cells were treated as in (**c**), and p53 and p21 protein levels were assessed by western blotting. Total p38 protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels

Figure 4d. Interestingly, although p53 and p21 levels were reduced in 501mel shTBX2 cells, it was not as dramatic as the effect seen in MCF-7 cells (Figure 4e). This suggested that TBX2 has a more important role in the p53 response in MCF-7 cells, which may account for our data showing that there is a more dramatic increase in sensitivity when TBX2 is depleted in these cells.

TBX2 is essential for the cisplatin-induced activation and stabilisation of p53. As an intact p53 response is critical for conferring cisplatin resistance in MCF-7 cells[22] and the above results indicated that this response may require TBX2, we further explored the relationship between p53 and TBX2 in the MCF-7 cells. We speculated that TBX2 may be involved in promoting p53 protein stability as quantitative real-time PCR data showed that TBX2 does not affect p53 transcript levels (Figure 5a). Furthermore, the MG132 proteosome inhibitor prevented p53 degradation in both untreated shCtrl and shTBX2 cells suggesting that TBX2 was not involved in the basal turnover of p53 levels (Figure 5b). Importantly, in the presence of MG132, p53 levels further increased in cisplatin-treated shCtrl cells but not in shTBX2 cells (Figure 5b). This confirmed that TBX2 is required for the stabilisation of p53 protein levels in response to cisplatin. Taken together, these results show that TBX2 has an important role in regulating p53 stability in response to cisplatin-induced damage.

To confirm that knocking down TBX2 sensitises MCF-7 cells to cisplatin as a result of an abrogated p53 response, we examined the effect of silencing p53 on markers of mitotic catastrophe in cisplatin-treated MCF-7 cells. Briefly, before cisplatin treatment, cells were transiently transfected with a control siRNA (si-Ctrl) or a siRNA to p53 (si-p53) and levels of cyclin B1 and γ-H2AX compared by western blotting. The results show that the si-p53 used was efficient in silencing p53 and that in general it had no effect on p-CHK2 and TBX2 levels (Figure 5c). As expected, unlike the si-Ctrl cells, p53-depleted cells were unable to upregulate p21 levels in response to cisplatin treatment (Figure 5c). Importantly, cisplatin-treated si-p53 cells underwent mitotic catastrophe as at time points at which they express high levels of γ-H2AX they are also expressing cyclin B1 (Figure 5c). Thus, the effect of silencing p53 is analogous to that seen when TBX2 was depleted indicating that TBX2 confers cisplatin resistance in MCF-7 cells through the p53 pathway.



Discussion

The effective treatment of breast cancer remains a major challenge due to the recurrence of treatment-resistant tumours. This includes the use of platinum-based chemotherapy that fails owing to tumours acquiring the ability to repair the drug-induced DNA damage. The development of

Figure 5 Increased TBX2 protein levels confer cisplatin resistance via a p53-dependent pathway. (**a**) MCF-7 shCtrl and shTBX2 cells were treated with 10 μM cisplatin for 24, 48 and 72 h and TBX2 and p53 transcript levels were assessed by quantitative real-time PCR. Values were normalised to GUSB mRNA levels. Error bars represent the S.E. of the mean of three independent experiments. (**b**) MCF-7 shCtrl and shTBX2 cells were treated with 10 μM cisplatin in the presence of MG132 (20 μM) or its vehicle (DMSO). p53 protein levels were assessed by western blotting. Total p38 protein levels were used as a loading control. The expression of p53 was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels. (**c**) MCF-7 cells were transiently transfected with 5 nM control siRNA (si-Ctrl) or a siRNA to p53 (si-p53). Twenty-four hours after transfection, cells were treated with cisplatin as described for (**a**), and TBX2, p-CHK2 (Thr68), p53, p21, cyclin B1 and γ-H2AX protein levels were determined by western blot analyses. Total p38 protein levels were used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels

therapies that combine inducers of DNA damage with inhibitors of the DNA-damage repair process is therefore clearly needed. Here we show that targeting TBX2 in combination with cisplatin treatment sensitises MCF-7 cells to this widely used chemotherapeutic drug. We demonstrate that silencing TBX2 disrupts the ATM-CHK2-p53 signalling pathway, which results in an abrogation of an S-phase arrest and DNA repair. Cells consequently enter mitosis with damaged DNA and undergo mitotic catastrophe.

TBX2 overexpression has been linked to chemotherapeutic drug resistance by an, as yet, unknown mechanism. Previous studies have shown that the ectopic expression of TBX2 in immortalised fibroblasts and adrenal carcinoma cells leads to cisplatin and doxorubicin resistance, respectively.[20,21] Furthermore, TBX2 has also been shown to be amplified in breast cancers carrying BRCA1/2 mutations,[12] which are known to acquire resistance to cisplatin.[34,35] However, whether the overexpression of endogenous TBX2 is indeed directly involved in TBX2-driven cancers acquiring resistance to chemotherapeutic drugs has not yet been demonstrated. Here we show that when TBX2 is knocked down in a cisplatin-resistant breast cancer cell line, the cells become sensitive to the drug and undergo mitotic catastrophe. These results were reproduced in a metastatic melanoma cell line and are significant because mitotic catastrophe is a preferred mechanism of cell death for chemotherapeutic agents because, unlike other checkpoints, the mitotic checkpoint is often functional in cancer cells and thus triggering this checkpoint guarantees cancer cell death.[36,37] Indeed, small-molecule inhibitors of CHK1 and KSP/Eg5, which promote mitotic catastrophe, are currently in preclinical and phase I clinical trials.[37]

Chemotherapeutic drugs that function by inducing DNA damage frequently fail due to tumours acquiring the ability to repair the DNA damage and therefore escaping cell death. The ATM-CHK2-p53 signalling cascade has been shown to regulate cisplatin-induced DNA damage repair and is thus an attractive pathway to disrupt in order to increase the efficacy of this drug.[9–11] In this study, we show that shTBX2 MCF-7 and 501mel cells treated with cisplatin undergo mitotic catastrophe because they lack an S-phase arrest resulting in them entering mitosis with damaged DNA. As the tumour suppressor and its target p21 are well-known inducers of this arrest, we conclude that the absence of a p53-p21 response in shTBX2 cells is responsible for this effect.[8] Importantly, in response to DNA damage, the p53 protein is stabilised by phosphorylation events involving the DNA structure checkpoint kinase, CHK2, which we show is not active in shTBX2 cells treated with cisplatin. We therefore speculate that the DNA repair response is disrupted is shTBX2 cells owing to prevention of CHK2 activation. These results are consistent with reported observations that the inhibition of CHK2, with either a chemical inhibitor or a dominant-negative expression construct, sensitises cancer cells to cisplatin and leads to mitotic catastrophe in doxorubicin-treated cells.[38,39]

Similar to our results in MCF-7 shTBX2 cells, we show that p53 depletion in combination with cisplatin, results in a lack of DNA repair and an induction of mitotic catastrophe. This significant finding is in line with several reports implicating wild-type p53 in drug resistance. For example, Yazlovitskaya *et al.*[40] demonstrated that cisplatin resistance was associated with prolonged stabilisation and accumulation of wild-type p53 in ovarian carcinoma cells.[40] Likewise, the head and neck squamous cell carcinoma cell lines, which were originally cisplatin sensitive, became resistant to this drug as a result of gain of p53 function.[41] In addition, whereas human cells harbouring wild-type p53 were able to successfully remove cisplatin-induced DNA cross-links, those with mutant p53 were unable to do so.[42] Importantly, Fan *et al.*[22] reported in 1995 that the disruption of wild-type p53 function sensitises MCF-7 cells to cisplatin, and Sangster-Guity *et al.*[43] have shown more recently that ATR-related cisplatin resistance in human colorectal cancer cells is dependent on p53.[22,44]

Data from the current study suggest that TBX2 does not repress basal or stimulated p21 expression. When TBX2 was silenced, p21 levels either remained unchanged in MCF-7 cells or were repressed in 501mel cells and in response to cisplatin-induced DNA damage, TBX2 and p21 protein levels increased simultaneously in both cell lines. Although this appears to conflict with findings that have demonstrated that TBX2 directly represses p21 *in vivo* and *in vitro*,[17,18,44] there are several studies that have reported on different responses of p21 expression to altered TBX2/Tbx2 levels. For example, when Redmond *et al.*[16] inhibited TBX2 using a dominant-negative approach, they observed a delayed increase in p21 protein levels implying an indirect regulation in MCF-7 cells.[16] On the other hand, two studies have shown no changes in p21 levels when TBX2/Tbx2 was either overexpressed in normal human lung fibroblasts[20] or knocked out in mice.[45] Given the involvement of p21 in multiple cellular processes ranging from differentiation to cell cycle regulation and cell death, one would expect it to be regulated by many factors and therefore it is unlikely that TBX2 would repress p21 in all cellular contexts.[46] This is perhaps best illustrated by observations that although p21 is a very well-described p53 target it is, as also demonstrated in the current study, regulated by p53-independent mechanisms.[46–48]

The current study demonstrates that in cisplatin-treated cells silencing TBX2 disrupts the ATM-CHK2-p53 axis, and based on our data we postulate that TBX2 is required for activating CHK2, which phosphorylates p53 leading to its

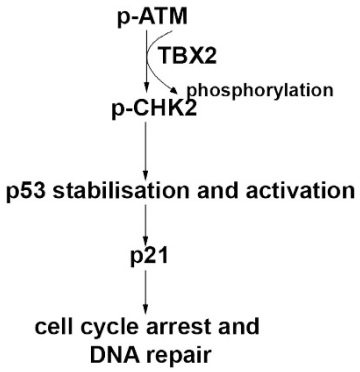


Figure 6 Proposed model showing the role of TBX2 in conferring drug resistance to DNA-damaging agents. TBX2 appears to be required for the activation of CHK2 and/or its maintenance ensuring the phosphorylation of p53 by CHK2 and hence its stability and transcriptional activity. Active p53 leads to the upregulation of p21 that triggers an S-phase arrest, DNA repair and cell survival

stabilisation and its ability to activate downstream targets (Figure 6). This is the first time that TBX2 is shown to have a role in the DNA-damage repair system and given the therapeutic impact of disabling CHK2 or p53 activation, the molecular detail of how TBX2 impacts on CHK2 activation warrants closer examination. This study provides compelling evidence in support of targeting TBX2 either alone or in combination with cisplatin as a viable option for treatment of TBX2-driven cancers. This is a particularly attractive possibility as TBX2 has no known function in normal adult tissues and its inhibition is therefore less likely to compromise tissue function as may be expected for inhibiting CHK2 or p53.

Materials and Methods

Cell culture and treatments. Human MCF-7 breast adenocarcinoma cells and 501mel human metastatic melanoma cells were maintained in RPMI 1640 medium (Highveld Biological, Johannesburg, South Africa) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in an atmosphere of 95% air/5% CO_2, 65% humidity. In order to induce DNA damage, cisplatin (Pfizer, Sandton, South Africa) was directly added to the medium to a final concentration of 10 μM (MCF-7) or 5 μM (501mel) for the time course indicated. The MCF-7 shCtrl, MCF-7 shTBX2, 501mel shCtrl and 501mel shTBX2 cells were previously described[13] and cultured as for the parental cells.

Small interfering RNA. Suppression of p53 cellular expression was achieved by an siRNA (small interfering RNA) that specifically targets p53 mRNA (Qiagen, Valencia, CA, USA). MCF-7 cells were transfected with 5 nM anti-p53 siRNA or a control (non-silencing) siRNA (Qiagen) using HiPerFect (Qiagen) according to the manufacturer's instructions.

Clonogenic survival assay. Cells were precultured and treated with the indicated concentration of cisplatin for 24 h, washed, collected and replated at 200 cells (MCF-7) or 100 cells (501mel)/ml in six-well plates. MCF cells were allowed to grow for 28 days and 501mel for 14 days, and surviving cells were fixed and stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence microscopy. Cells grown on glass coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature (RT) before permeabilisation with 0.2% Triton X-100 in PBS for 10 min at RT. Slides were incubated over night with rabbit polyclonal anti-phospho-H2AX (ser139) (γ-H2AX) (Cell Signaling Technology Inc., Danvers, MA, USA) at a dilution of 1 : 500, then incubated with the appropriate secondary antibody coupled to Cy3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at a 1 : 1000 dilution. Cells were mounted using Mowiol mounting medium and examined by fluorescence or confocal microscopy (Zeiss LSM 510 Meta with NLO, Software: ZEN 2009, Lasers: Argon 488 green, solid-state laser: 561 nm Red, MaiTai 2 photon laser: 750 nm for DAPI/Hoechst, Jena, Germany).

Western blot analysis. Whole-protein extracts were separated on 6–15% SDS-polyacrylamide gels and transferred to Hybond C (Amersham, Buckinghamshire, UK) nitrocellulose membranes. Membranes were probed with appropriate primary antibodies followed by a peroxidase-conjugated anti-mouse, anti-goat or anti-rabbit antibody (1 : 5000) and visualised by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies and appropriate dilutions were as follows: goat polyclonal anti-Tbx2 (1 : 1000); mouse monoclonal anti-ATM (1 : 500), anti-p53 (1 : 1000); rabbit polyclonal anti-p21 (1 : 1000), anti-PARP-1/2 (1 : 1000) and anti-cyclin B1(1 : 1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal anti-p38 (1 : 5000) (Sigma); rabbit polyclonal anti-phospho-H2AX (ser139) (γ-H2AX) (1 : 1000); rabbit monoclonal anti-phospho-CHK2(thr68) (1 : 1000); and mouse monoclonal anti-phospho-ATM (ser1981) (1 : 1000) (Cell Signaling Technology Inc.).

Flow cytometry. Cells were collected by trypsinisation, washed twice with PBS, suspended in 2 ml of cold PBS and fixed in 8 ml of 70% cold ethanol for at least 30 min at -20 °C. Cells were pelleted by centrifugation, washed twice with PBS and treated for 15 min at 37 °C with 50 μg/ml RNase A. Cells were stained at RT with propidium iodide (PI) solution (2 mM $MgCl_2$, 10 mM Pipes buffer, 0.1 M NaCl, 0.1% Triton X-100, 0.01 mg/ml PI) and subjected to analysis in a Beckman FACSCalibur flow cytometer.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from cells using the RNeasy Plus Mini kit (Qiagen). Reverse transcription of RNA (1 μg) was performed according to the manufacturer's instructions using the InProm-IITM reverse transcription system (A3800; Promega, Madison, WI, USA). Quantitative real-time PCR was conducted on an Applied Biosystems (Carlsbad, CA, USA) StepOne Plus thermal cycler using 2x SYBR green master mix (Applied Biosystems), a final concentration of 0.3 μM of each primer and 2 μl of cDNA in a total volume of 10 μl. PCR cycle parameters were: denaturation for 15 min at 95 °C, combined annealing and extension for 35 cycles at 60 °C for 1 min. Each DNA sample was quantified in triplicate and a negative control without cDNA template was run with every assay to assess the overall specificity. Melting curve analyses were carried out to ensure product specificity. Relative mRNA expression levels were normalised to glucuronidase beta (GUSB) using the $2^{-\Delta\Delta Ct}$ method. Primers used to amplify human TBX2 (catalogue no. QT00091266), human p53 (catalogue no. QT00060235) and human GUSB (catalogue no. QT00046046) were purchased from Qiagen.

Conflict of Interest

The authors declare no conflict of interest.

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