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## **FOXP3 and FOXP3-regulated microRNAs suppress the expression of SATB1 in breast cancer cell lines**

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## Abstract

The transcription factor FOXP3 has been identified as a tumour suppressor in breast and prostate epithelia, but little is known about its specific mechanism of action. We have identified a feed-forward regulatory loop in which FOXP3 suppresses the expression of the oncogene *SATB1*. In particular, we demonstrate that *SATB1* is not only a direct target of FOXP3 repression but that FOXP3 also induces two microRNAs; miR-7 and miR-155, which specifically target the 3' UTR of *SATB1* to further regulate its expression. We conclude that FOXP3-regulated microRNAs form part of the mechanism by which FOXP3 prevents the transformation of healthy breast epithelium to a cancerous phenotype. Approaches aimed at restoring FOXP3 function and the miRs it regulates could help provide new approaches to target breast cancer.

## Introduction

The X-linked gene *FOXP3* is a member of the Forkhead/winged helix family of transcription factors which was first identified as essential for the formation and function of T regulatory (Treg) cells. Treg are a rare subset of T cells responsible for preventing inappropriate immune responses and promoting immune tolerance (Feuerer *et al.*, 2009; Fontenot *et al.*, 2003; Josefowicz and Rudensky, 2009; Marson *et al.*, 2007). Although originally thought to be T cell-restricted, *FOXP3* expression appears widespread in normal epithelia and aberrant in solid tumours, suggesting that FOXP3 can function as a tumour suppressor (Chen *et al.*, 2008; Ladoire *et al.*, 2011; Martin *et al.*, 2010). Compelling evidence for this comes from the observation that female heterozygous *Foxp3* knockout mice have a significant age-dependent increase in the incidence of spontaneous cancer, in particular mammary cancer. Critically, tumour cells, but not adjacent normal breast tissue from these mice showed skewed X-inactivation, resulting in the exclusive loss of the WT *Foxp3* allele in the cancer (Zuo *et al.*, 2007b). Further evidence for a tumour suppressor role comes from prostate-specific ablation of FOXP3 by conditional gene targeting, which leads to prostate hyperplasia and precancerous lesions (Wang *et al.*, 2009). FOXP3 also appears to have tumour suppressor functions in human epithelial cells as several studies report a high frequency of somatic mutations and deletions of FOXP3 in human breast, ovary and prostate tumour samples (Zuo *et al.*, 2007a; Zuo *et al.*, 2007b). In addition, the reintroduction of FOXP3 into breast or prostate cancer cell lines has growth inhibitory and pro-apoptotic effects on these cancer cell lines *in vitro* and *in vivo* (Wang *et al.*, 2009; Zhang *et al.*, 2006; Zuo *et al.*, 2007a).

FOXP3 is able to function as a transcriptional repressor or activator in both Treg (Marson *et al.*, 2007; Sadlon *et al.*, 2010; Zheng *et al.*, 2007) and epithelial cells. For example, FOXP3 directly represses the *ERBB2* and *SKP2* oncogenes whilst maintaining *p21* tumour suppressor expression in breast epithelium (Liu *et al.*, 2009a; Zuo *et al.*, 2007a; Zuo *et al.*, 2007b). In prostate cells, FOXP3 has also been shown to directly repress c-myc transcription (Wang *et al.*, 2009), an oncogene frequently over-expressed in many types of human cancer (Wolfer *et al.*, 2010). More recently, induction of *FOXP3* by p53 following DNA damage in breast epithelial cells has been reported with increased FOXP3 levels contributing to the growth suppressive activity of the p53 pathway (Jung *et al.*, 2010). Together these data suggest that FOXP3 is itself regulated by tumour suppressors and it controls key oncogenes and tumour suppressors in epithelial cells, although the mechanisms and pathways have yet to be fully elucidated. This raises the question as to whether *FOXP3* is linked to other genes involved in maintaining breast epithelium.

Whilst the targets of FOXP3 in epithelial cells are still largely unknown, genome-wide chromatin immunoprecipitation (ChIP-on-chip) studies in human and mouse Treg cells have identified a large number of potential FOXP3 target genes, including a number of loci encoding microRNAs, (Marson *et al.*, 2007; Sadlon *et al.*, 2010; Zheng *et al.*, 2007). The extent to which FOXP3 regulates common or unique sets of genes in these two different cell types is not currently known, however, the identification of microRNAs as potential targets for FOXP3 regulation is of clear interest given their regulatory role in both normal and disease tissue. MicroRNAs (miRs) are a class of small non-coding RNAs that modulate gene expression by binding to the 3'UTR of target genes, resulting in translational repression and increased mRNA degradation (Bartel, 2009; Filipowicz *et al.*, 2008). In breast cancer, expression profiling has demonstrated clear differences in the expression of specific miRs

between normal and primary tumour tissue, with miRs identified that are both up and downregulated (Iorio *et al.*, 2005; Volinia *et al.*, 2006). The mechanisms and consequences of miR deregulation within a cancer cell are still being determined, although it is likely that changes in the levels and activity of transcriptional regulators and miR processing machinery, coupled with alterations in miR copy number, will contribute to the overall deregulation of miR expression (Selcuklu *et al.*, 2009; Zhang *et al.*, 2006). There is now compelling evidence for the participation of miR and transcription factor combinations in feed-back and feed-forward loops (Bartel, 2009; Brosh *et al.*, 2008; Re *et al.*, 2009; Tsang *et al.*, 2007), and such microRNA/transcription factor feed-forward loops have been shown to enhance the robustness and responsiveness of a regulatory network (Filipowicz *et al.*, 2008).

Special AT-rich Binding Protein 1 (*SATB1*), an oncogene involved in the promotion of breast tumour growth and metastasis (Han *et al.*, 2008; Li *et al.*, 2010a; Li *et al.*, 2010b), is expressed in a significant proportion of primary human epithelial breast tumours and is an independent prognostic factor for breast cancer (Han *et al.*, 2008). *SATB1* is expressed in a number of metastatic breast cancer cell lines but not in non-invasive cell lines (Han *et al.*, 2008), and furthermore, knockdown of *SATB1* in these aggressive breast cancer cells significantly inhibited the growth of tumours in both *in vitro* and *in vivo* models of metastasis, providing strong evidence of its essential role in cancer progression (Han *et al.*, 2008; Richon, 2008). *SATB1* is a DNA binding protein that functions as a global regulator of transcription through diverse mechanisms which include the maintenance of specific chromatin architecture, such as the creation of chromatin loop domains by selectively tethering specific matrix attachment regions (MARs) to the nuclear matrix, and the recruitment of chromatin modifying complexes to these anchor points (Cai *et al.*, 2003; Galande *et al.*, 2007; Yasui *et al.*, 2002).

Here we show that FOXP3 over-expression in the BT549 breast cancer cell line can suppress SATB1 mRNA and protein levels. Specifically we show that FOXP3 together with the FOXP3-induced miRs hsa-miR-7 (miR-7) and hsa-miR-155 (miR-155) form a feed forward regulatory loop to suppress SATB1. We therefore propose that restoration of this regulation may provide a new approach in the treatment of breast cancer.

## Methods

### Cell lines

BT549 cells (ATCC, Manassas, Virginia USA) were cultured in RPMI (Thermo Scientific, Waltham USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, Missouri USA) and 1% Penicillin/streptomycin solution (Sigma-Aldrich). MDA-MB-231 cells (ATCC) and HEK 293T cells (ATCC) were cultured in Dulbecco's modified Eagle Medium (DMEM; Thermo Scientific) supplemented with 10% FBS and 1% Pen/Strep. All cell lines were maintained at 37°C and 5% CO<sub>2</sub>.

### Lentiviral production and transduction

The lentivirus construct pLV411-FOXP3 described previously (Brown et al 2010) encodes full length human FOXP3 transcribed from the Elongation factor 1a promoter, whilst enhanced green fluorescence protein (eGFP) is expressed from an internal ribosome entry site (IRES). Lentivirus preparations were generated as previously described (Barry *et al.*, 2001). Briefly, HEK 293T (6x10<sup>6</sup> /T75cm<sup>2</sup> flask) cells were transfected with lentiviral transfer vector and packaging vectors by reverse transfection using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, California USA). A total of 30ug of DNA was used consisting of expression vectors for Gag/Pol (7.6ug); Rev (6.4ug); VSV-G (3.6ug) and transfer vector containing the gene of interest (12.4ug). The following day medium was replaced and virus harvested 72 hours post transfection. Medium was passed through a 0.45um filter to remove cellular debris and stored at -80°C prior to use. The transfection efficiency of each packaging reaction was determined by limiting dilution transduction of HEK 293T cells. Transduced cells were identified with flow cytometric detection of GFP-positive cells with viral titres determined by calculating the percentage of GFP-positive HEK 293T cells/cell number x dilution. Breast cancer cell lines were transduced with lentivirus at a multiplicity of infection



(MOI) of 2, and sorted for GFP on day 4. Sorted cells were cultured and used for further experiments.

#### Cell proliferation assay

The proliferative capacity of cells was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit as per the manufacturer's recommendations (Promega, Madison, Wisconsin USA). To measure viability and proliferation of FOXP3-transduced and control cell lines cells were plated at  $1 \times 10^4$  cells in 100ul of the appropriate media in a 96 well plate in triplicate and the cell proliferation assay performed over a 5 day period. Following the addition of CellTiter 96 AQueous One Solution Reagent to triplicate wells the plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 1 hour prior to the absorbance at 490nm being determined.

#### Quantitative Real Time PCR

Total RNA was extracted from all cell lines using the miRNeasy kit (Qiagen, Hilden Germany) and subsequently converted to cDNA using the Quantitect Reverse Transcription kit (Qiagen). Semi-Quantitative RT-PCR was performed using the KAPA SYBR Fast Universal qPCR kit (KAPA Biosystems, Cambridge, Massachusetts USA) in triplicate. PCR reactions were performed on a Corbett real time PCR machine (RotorGene 6000). Results from three independent experiments were analysed using Rotor-Gene 6000 software and normalised to the expression of reference transcript ribosomal protein L13a (RPL13a). The primers used in this study are listed in supplementary table S1.

### MicroRNA RT-PCR

Target-specific reverse transcription was performed using the Taqman microRNA Reverse Transcription kit (Applied Biosystems, Foster City, California USA) and Taqman microRNA assay probes for the appropriate miRs (Applied Biosystems, for miR-24, -7, -101 and -155). Detection of the mature microRNA forms were performed using specific Taqman microRNA assays in the presence of Taqman Universal PCR Mastermix (Applied Biosystems). Assays were performed in triplicate on a Corbett Real-time machine (Rotorgene 6000) with miR-24 used as a reference miR for normalisation. Results from three independent assays were analysed using Rotor-Gene 6000 software.

### Western Blot and Immunodetection

Total cell lysate was extracted from cells using RIPA buffer (1% NP-40, 0.1% SDS, 0.1% Na Deoxycholate, 100mM NaCl, 2.5mM EDTA, 2.5mM EGTA, 50mM HEPES pH 7.4, 1mM Pefablock, 1x complete protease inhibitor cocktail). Protein estimation was performed using a Dc assay kit (Bio-Rad, Hercules, California USA). Western blots were performed with total protein (30ug) as per the BD Biosciences protocol for western blotting with monoclonal antibodies. Nitrocellulose membranes were probed with a mouse anti-SATB1 monoclonal antibody (BD biosciences, Franklin Lakes, New Jersey USA; Clone 14/SATB1). Detection of proteins of interest was performed using West Dura Chemiluminescent detection kit (Pierce). Band intensities were quantitated using ImageJ 1.43 (<http://rsbweb.nih.gov/ij/>) after scanning on a G: BOX iChemi imager (Syngene, Cambridge UK). As a loading control filters were stripped as per instructions for Western Blot Stripping Solution (Alpha Diagnostics, Texas USA) and subsequently re-probed with a polyclonal rabbit anti- $\alpha$ -tubulin antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania USA).

### Luciferase Constructs and Assays

Promoter reporter constructs to determine the FOXP3-responsiveness of FOXP3 binding regions identified in the human *SATB1* gene were constructed as follows; FOXP3 binding regions were cloned into the pGL4.24 vector downstream of a minP element and upstream of a destabilised firefly luciferase. To measure the response to FOXP3, the *SATB1* promoter reporter constructs were transfected into parental, *FOXP3*-expressing or GFP control lines together with pGL4.74 renilla luciferase construct using Lipofectamine 2000. Luciferase activity was assessed 24 hours after transfection using a Dual Luciferase kit (Promega) as per the manufacturer's instructions in a Veritas luminescent plate reader (Promega).

To determine the effect of specific microRNAs on *SATB1* expression and to identify functional microRNA binding sites in the 3'UTR of *SATB1* (as shown in Figure 1b) the 3'UTR of *SATB1* was amplified by PCR and cloned into the psiCHECK-2 vector (Promega). Mutations in putative microRNA binding sites were performed using the KAPA HiFi HotStart mutagenesis PCR protocol (Kapa Biosystems, Boston, Massachusetts USA). HEK 293T cells or breast cancer cell lines were then simultaneously transfected with psiCHECK-2 constructs and synthetic pre-miR molecules (Ambion) using the miR-optimised Hiperfect transfection reagent (Qiagen). Transfections were performed in triplicate and luciferase activity was measured 24 hours later. To determine the effect of pre-miR overexpression on endogenous *SATB1* levels, breast cancer cell lines (BT549 and MDA-MB-231) were transfected with pre-miR molecules using Hiperfect transfection reagent and RNA or protein was isolated as described above 24 or 48 hours post-transfection respectively.

### MicroRNA Knockdown

Peptide Nucleic Acid (PNA) microRNA inhibitors for miR-7 and -155 and a control non-targeting PNA were purchased from Panagene (Daejeon, Korea). To measure knockdown of microRNAs in luciferase reporter assays, BT549 cell lines were transfected with reporter plasmids and the PNA inhibitors using Lipofectamine 2000 in a 96-well format and luciferase activity was determined 24 hours later.

## Results

### Identification of SATB1 as a target for a FOXP3-microRNA feed-forward regulatory loop

In a previous study we performed ChIP-on-chip experiments to identify genes potentially regulated by FOXP3 in human Treg cells (Sadlon *et al.*, 2010). Ingenuity Pathways Analysis of these genes indicates a statistically significant enrichment of genes associated with cancer, apoptosis, control of growth and proliferation (Supplementary Figure S1). We hypothesised that some of these targets may also be regulated by FOXP3 in breast epithelium. Furthermore, because a number of microRNAs were found to be regulated by FOXP3 in human Treg cells (Sadlon *et al.*, 2010), we searched the list of FOXP3 target genes for genes with a known role in cancer, apoptosis and control of growth that were also predicted to be targets of microRNAs that are regulated by FOXP3. Putative microRNA binding sites in the 3'UTRs of candidate FOXP3 target genes were first identified using the microRNA target prediction programs in miRGen (<http://www.diana.pcbi.upenn.edu/miRGen.html>). Then using the selection criteria that a candidate binding site must be found in the intersection of three separate prediction programs (miRanda, PicTar4 and TargetScan), we identified *SATB1* as a gene that has multiple binding sites for FOXP3 in its promoter region (Figure 1a) as well as predicted binding sites for the FOXP3-regulated miR-7 and miR-155 in its 3' UTR (Figure 1b).

### FOXP3 over expression reduces SATB1 levels in BT549

In order to determine if FOXP3 levels influence *SATB1* expression in breast cancer cells the lentiviral pLVEIG-FOXP3 vector was used to stably express human *FOXP3* in BT549 human breast cancer cells. BT549 cells have been demonstrated previously to express *SATB1* which is associated with their metastatic potential (Han *et al.*, 2008) and express low/negligible

levels of FOXP3 (Figure 2). Multiple independent transductions of BT549 cells with either a *FOXP3*-containing lentivirus or a control GFP-marked vector yielded cell lines in which long term stable expression of the vector was observed (Supplementary Figure S2). A reduction in proliferative capacity of the BT549 cell line was observed when *FOXP3* was stably over expressed (Supplementary Figure S3), and comparison of the GFP-transduced cells with the parental cell lines confirmed this reduction was not due to the effects of lentiviral integration. *FOXP3* over expression in BT549 cells was not associated with significant loss of viability.

The impact of *FOXP3* expression on *SATB1* levels in BT549 cells was then determined by RT-PCR and western blot analysis. *SATB1* mRNA levels were reduced by ~60% ( $p < 0.0001$ ,  $n = 3$  separate transduction pools) in BT549 cell lines in which *FOXP3* was over expressed compared to un-transduced BT549 cells (Figure 2a). In contrast, *SATB1* mRNA levels were not significantly altered when cells were transduced with a GFP-expressing control vector. The effect of FOXP3 on *SATB1* protein levels was confirmed by western blot (Figure 2b). Together these data indicate that FOXP3 is capable of repressing *SATB1* levels in breast cancer cell lines.

#### FOXP3 regulates *SATB1* transcription in BT549

FOXP3 Chromatin immunoprecipitation arrays (ChIP-on-chip) in Treg cells indicated several potential binding interactions in the *SATB1* locus (Figure 1a), suggesting that the *SATB1* promoter may be responsive to FOXP3. To test whether FOXP3 directly represses the *SATB1* promoter in breast cancer cells, we constructed *SATB1*-promoter luciferase reporter constructs. These were transfected into either control BT549 cells or BT549 cells expressing *FOXP3*. We examined the FOXP3-dependent regulation of two binding regions (BR-1 and BR-2) located -3kb and +0.5kb upstream and downstream from the *SATB1* transcription start

site (TSS) respectively. BT549 cells transfected with a *SATB1* promoter reporter containing either FOXP3 BR-1 or BR-2 showed significant repression in BT549-FOXP3 cells compared to BT549 or BT549-GFP cells (Fig. 3)(BR-1 ~75% repression,  $p < 0.0001$  and BR-2 ~40% repression  $p = 0.048$  respectively for  $n = 3$  independent experiments). Importantly, when the FOXP3 binding consensus sequences located in BR-1 or BR-2 were mutated the FOXP3-dependent repression of luciferase activity was abolished. In addition, no effect of FOXP3 expression was observed on a control promoter construct (psiCHECK control) in which luciferase transcription was driven by the HSV-TK promoter. Together these data indicate that FOXP3 is able to directly regulate the *SATB1* promoter in BT549 cells.

#### FOXP3-induced microRNA expression in BT549

We hypothesised that FOXP3 may act additionally via microRNAs to down-regulate *SATB1* in breast epithelial cells. In particular we focussed on miR-155 and miR-7 as these microRNAs have predicted binding sites located within the 3'UTR of *SATB1*. FOXP3 binding regions in close proximity to the loci encoding miR-155 and two of the three genomic loci encoding miR-7 (miR-7-1 and miR-7-2) have been previously identified (Sadlon *et al.*, 2010). To determine if FOXP3 can alter the expression level of these microRNAs, the relative levels of miR-7 and miR-155 in BT549 cells expressing pLVEIG *FOXP3* were compared to parental BT549 cells as determined by qRT-PCR. Relative to the parental BT549 cell line or cells transduced with the GFP control lentivirus, there were significantly elevated levels of miR-7 and miR-155 in the BT549-FOXP3 cells, suggesting that FOXP3 could regulate the expression of these microRNAs in breast epithelial cell lines (Figure 4).

### Downregulation of SATB1 by microRNAs -7 and -155

To determine whether miR-7 and miR-155 directly target *SATB1* in breast cancer cells, we constructed a luciferase reporter containing the *SATB1* 3'UTR. The reporter gene was co-transfected into BT549 cells along with pre-miRs for miR-7, miR-155, a combination of both, or a non-targeting random sequence control pre-miR. When tested over a range of concentrations, a significant dose-dependent reduction in the levels of *SATB1* 3'UTR reporter activity was observed for miR-7 alone (60% reduction,  $p=0.03$ ), miR-155 alone (55% reduction,  $p=0.03$ ), and the combination of the two miRs (70% reduction,  $p<0.0001$ ), but not the non-targeting control miR (Figure 5a). No miR-dependent reduction in the renilla activity of a control reporter construct lacking the *SATB1* 3'UTR sequences was observed (Figure 5b). Sequence mutations were then introduced to disrupt the miR seed sequences for miR-7 and miR-155 either alone or in combination in the *SATB1* 3'UTR reporter constructs (Figure 1b). These constructs were co-transfected into BT549 cells with a control miR, pre-miR-7 or pre-miR-155. Mutation of either of the putative miR binding sites within the *SATB1* 3'UTR abolished the ability of the corresponding pre-miR to down-regulate renilla luciferase activity whilst mutation of both miR seed sites prevented the down-regulation of renilla activity by either miR (Figure 5b). We observed similar miR-7 and miR-155 down-regulation of *SATB1* 3'UTR reporter construct activity in MDA-MB-231 cells, a second breast cancer cell line known to express high levels of SATB1 protein (Han et al 2008) (Supplementary Figure S4). These observations suggest that the *SATB1* 3'UTR contains functional binding sites for miR-7 and miR-155 and together the level of these two miRs contribute to the overall level of SATB1 within the cell.



### Endogenous miR-7 and miR-155 regulate SATB1 levels in BT549 cells

To verify that the endogenous miR-7 and miR-155 in BT549 cells can regulate SATB1, we used peptide nucleic acid (PNA) inhibitor molecules against miR-7 or miR-155 to block miR-7 and miR-155 activity respectively. BT549 cells expressing either FOXP3 or GFP alone were transiently co-transfected with psiCHECK/*SATB1* 3'UTR reporter constructs and either a control PNA miR inhibitor or specific miR-7 and miR-155 PNA inhibitors. In cells expressing GFP alone, co-transfection with PNA inhibitors (100-200nM) to either miR-7 or miR-155 resulted in a 30-40% increase in reporter activity ( $n=3$   $p<0.0001$ ) whereas no significant effect on renilla luciferase activity was observed with the control PNA inhibitor at these concentrations (Figure 6). In BT549 cells expressing *FOXP3*, a 45% reduction in the *SATB1* 3'UTR reporter activity relative to BT549 cells expressing GFP alone was apparent in the absence of PNA inhibitors ( $n=3$   $p<0.0001$ ), consistent with the FOXP3-dependent increase in the expression of these miRs observed in these cells (Figure 4). Co-transfection of these cells with 200nM miR-7 or miR-155 PNA inhibitors caused a 30-40% ( $p=0.004$ ) dose-dependent PNA-specific reversal of the inhibition of the *SATB1* 3'UTR reporter constructs compared with the BT549 GFP no miR inhibitor control. When compared with the BT549 FOXP3 no miR-i control this represents a 50-60% increase in SATB1 reporter activity. No significant effects on the reporter activity were observed when FOXP3-expressing cells were co-transfected with control PNA inhibitors. Similar increases in reporter activity were observed when parental BT549 cells were treated with miR-7 and miR-155 inhibitor, but not control PNA inhibitor (Supplementary Figure S5).

### Repression of endogenous SATB1 by microRNAs -7 and -155

To determine if miR-7 and miR-155 could repress endogenous *SATB1*, the parental BT549 cell line, GFP-transduced BT549 cells and FOXP3-overexpressing BT549 cells were

transfected either with a control pre-miR, pre-miR-7 or pre-miR-155. Transfection of the parental and GFP cell lines with either pre-miR-7 or pre-miR-155 caused a significant reduction in *SATB1* mRNA levels compared to control pre-miR transduced cells (35-38%  $p=6.35 \times 10^{-5}$  and 35-45%  $p=1.4 \times 10^{-9}$  reduction respectively) (Figure 7a). In FOXP3-transduced cells transfected with the control pre-miR, endogenous *SATB1* mRNA levels were decreased 45-50% ( $p=1.03 \times 10^{-12}$ ) compared to control pre-miR transfected parental or GFP transduced cell lines. This was considered to be most likely due to the combination of FOXP3-dependent repression of *SATB1* transcription and up-regulation of endogenous miR-7 and miR-155 levels within these cells. Importantly, when pre-miR-7 or pre-miR-155 were transfected into *FOXP3*-expressing cells a further significant decrease in *SATB1* mRNA levels was observed with a 40-43% ( $=3.12 \times 10^{-5}$ ) and 70-72% ( $p=1.35 \times 10^{-8}$ ) reduction in *SATB1* mRNA observed in miR-7 and miR-155 transfected cells respectively. When compared to the control *SATB1* expression levels in BT549 cells, the additive effects of FOXP3 and miR-7 or miR-155 account for a 70-80% reduction in *SATB1* expression. These findings were confirmed at the protein level by western blot assay of SATB1 protein expression (Figure 7b). To quantitate the effect of FOXP3 and miR-7 and miR-155 on SATB1 protein levels, three independent western blots were analysed and the relative level of SATB1 was normalised to alpha tubulin (Figure 7c). A significant miR-7 and miR-155-dependent reduction in SATB1 protein levels was observed in all three cell lines. Additionally, FOXP3 expression alone caused a reduction in SATB1 protein levels, with further reduction in SATB1 levels detected when these cells were also transfected with pre-miR-7 or pre-miR-155. Together these findings suggest that FOXP3 acts to negatively regulate SATB1 levels both directly, through the repression of *SATB1* transcription, and indirectly through the induction of miR-7 and miR-155 that then target the 3'UTR of *SATB1* mRNA.

## Discussion

Recently the transcription factor FOXP3, a master regulator of Treg cells, has been proposed to function as a tumour suppressor within breast and prostate epithelial cells (Wang *et al.*, 2009; Zuo *et al.*, 2007b), although the molecular pathways and targets it regulates are yet to be explored in detail. In Treg cells FOXP3 orchestrates its transcriptional program both by directly binding to gene regulatory regions and also indirectly by controlling other regulators such as transcription factors and microRNAs (Gavin *et al.*, 2007; Hill *et al.*, 2007; Sadlon *et al.*, 2010; Williams and Rudensky, 2007; Zheng *et al.*, 2007). In this work we demonstrate that FOXP3 can also carry out its tumour suppressor function in epithelial cells by direct and indirect mechanisms. Specifically, we show that in breast cancer cell lines *SATB1* is repressed by FOXP3 both directly, by FOXP3 binding to regulatory regions within the *SATB1* gene, and indirectly through FOXP3-induction of two miRs; miR-7 and miR-155, which themselves target the 3'UTR of *SATB1*. This suggests that part of FOXP3 tumour suppressor activity within normal breast may be via the suppression of *SATB1* expression. *SATB1* has recently been proposed to be an important mediator of epithelial-mesenchymal transitions (EMT) and metastasis in breast cancer. Furthermore, *SATB1* protein expression has been linked to poor prognosis in a large cohort of breast cancer patients (Han *et al.*, 2008; Hanker *et al.*, 2010; Kohwi-Shigematsu *et al.*, 2010). Although there are contradictory reports on the prognostic value of *SATB1* expression in breast cancer based upon the assessment of transcript levels (Hanker *et al.*, 2010; Iorns *et al.*, 2010), contamination of *SATB1* signals from other cell types such as activated stromal cells or infiltrating T lymphocytes may explain this (Kohwi-Shigematsu *et al.*, 2010). Critically, Han *et al.* scored *SATB1* protein expression only in tumour cells (Han *et al.*, 2008), which gave a strong prognostic correlation. Three recent studies indicated that high *SATB1* mRNA levels

correlated with advanced stages of cancer and poor prognosis in breast (Patani *et al.*, 2009) and gastric cancer (Cheng *et al.*, 2010; Lu *et al.*, 2010). *SATB1* expression has also recently been linked to the acquisition of multidrug resistance in MCF7 cells (Li *et al.*, 2010b) and in chemotherapy induced-EMT within breast cancer (Li *et al.*, 2010a). Together these findings are consistent with SATB1 playing an important role in cancer.

Little is known about the regulation of SATB1 levels and, in particular, the mechanism by which *SATB1* expression is induced in breast cancer. We show for the first time that FOXP3 and FOXP3-induced miRs are able to form a feed-forward loop to control *SATB1* expression in breast cancer cells. Potentially, the up-regulation of FOXP3 or FOXP3-regulated miRs may provide a new therapeutic opportunity for the treatment of breast cancer. As a consequence of *FOXP3* being subject to random X-inactivation, only a single allele of *FOXP3* needs to be mutated to inactivate FOXP3 function (Liu *et al.*, 2010). Indeed thus far the majority of mutations and deletions of *FOXP3* found in breast cancer samples are heterozygous, with only the *FOXP3* allele on the active X-chromosome mutated (Zuo *et al.*, 2007a; Zuo *et al.*, 2007b). This raises the possibility of re-activating the wild type allele as a potential cancer therapy as recently demonstrated in normal and malignant mammary cells in vitro (Liu *et al.*, 2009b).

Our findings establish a link between *FOXP3* expression and the suppression of metastatic potential, at least in part through the regulation of *SATB1*. Other mechanisms also participate in the suppression of *SATB1* within breast epithelial cells, including induction of other miRs such as miR-488 (Li *et al.*, 2010a). *FOXP3* loss appears to occur more frequently in primary breast cancer cells and breast cancer cell lines than does *SATB1* induction (Han *et al.*, 2008; Zuo *et al.*, 2007b), suggesting that FOXP3 may also regulate other genes involved in cancer. Together, these findings indicate that *SATB1* expression is tightly regulated within breast epithelia both through transcriptional control and post-transcriptionally through microRNAs.

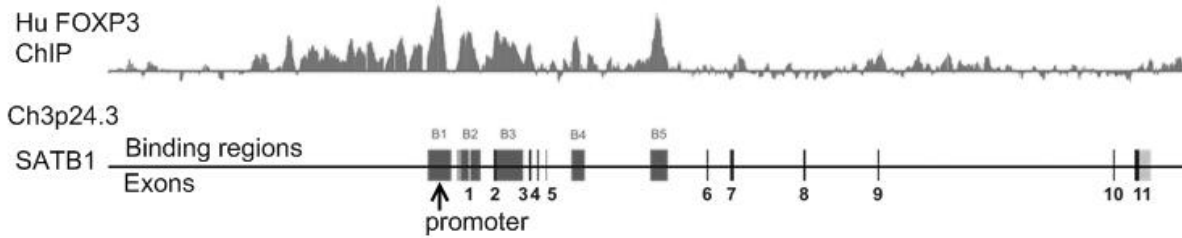
The finding that FOXP3 can regulate miR-7 and miR-155 in breast cancer cell lines suggests that aberrant expression of miRs may be an important consequence of loss of FOXP3 activity in primary tumour cells. The aberrant expression of other miRs in cancer cells has also been recently linked to the loss and/or oncogenic activation of transcription factors with tumour suppressor function or oncogenic potential respectively (Bommer *et al.*, 2007; O'Donnell *et al.*, 2005; Reddy *et al.*, 2008; Sander *et al.*, 2008). The exact role for the FOXP3-induced miRs identified in our study in normal and cancerous epithelial cells is yet to be determined, as each miR can potentially target multiple transcripts (Farazi *et al.*, 2010). However, experimentally validated miR-7 targets including growth factor receptors and signalling molecules including EGFR, PAK1, RAF-1, IRS1 and IRS2 are frequently up-regulated in cancers (Kefas *et al.*, 2008; Pogribny *et al.*, 2010; Reddy *et al.*, 2008), supporting a role for miR-7 in FOXP3 tumour suppressor function. We have also observed reduced mRNA levels for the miR-7 targets *EGFR*, *PAK1* and *RAF-1* in *FOXP3* transduced BT549 cells correlating with the increased miR-7 levels (Supplementary Figure S6), suggesting that in addition to suppressing *SATB1*, miR-7 may also suppress other important oncogenes within these cells. Although increased miR-7 levels have been associated with aggressiveness in estrogen receptor-positive (ER+) breast cancers (Foekens *et al.*, 2008), the recent finding that miR inhibitory effects are generally less potent in ER+ breast cancers provides a possible explanation for this apparent contradiction (Cheng *et al.*, 2009). Clearly, further work is required to determine the role miR-7 has in breast cancer pathogenesis. Similarly, the role of miR-155 needs further clarification. MiR-155 is over-expressed in many cancers including breast cancer where it has been shown to promote growth and cell survival (Blenkiron *et al.*, 2007; Iorio *et al.*, 2005; Kong *et al.*, 2010), which is apparently at odds with its ability to target the *SATB1* oncogene as identified in this study, and inconsistent with it playing a role in FOXP3 tumour suppressor function. These seemingly contradictory findings may reflect

the difference between the function of a specific miR in the context of a cancerous cell compared to a normal cell. Presumably within a normal epithelial cell, as observed for Treg cells (Kohlhaas *et al.*, 2009; Lu *et al.*, 2009), FOXP3 contributes to a steady state level of miR-155 required for normal cellular and/or tissue homeostasis. However within the context of multiple changes that occur within a cancerous cell, increased levels of miR-155 may have a deleterious effect.

We propose that re-expression of *FOXP3* in cancer cells, in which *FOXP3* expression has been lost, will result in FOXP3-mediated up-regulation of miR-155, miR-7 and other miRs, restoring the regulation which normally occurs in non-malignant cells. In summary, we have identified a feed-forward loop involving FOXP3 and FOXP3-induced microRNAs and demonstrated that the oncogene *SATB1* is a target for suppression by this loop (Figure 8). The identification of other potential targets of FOXP3-dependent feed-forward regulatory loops may provide additional candidates for intervention in breast cancer.

Figure 1 Potential FOXP3 and FOXP3-regulated miR binding sites within the *SATB1* gene and mRNA

A)



B)

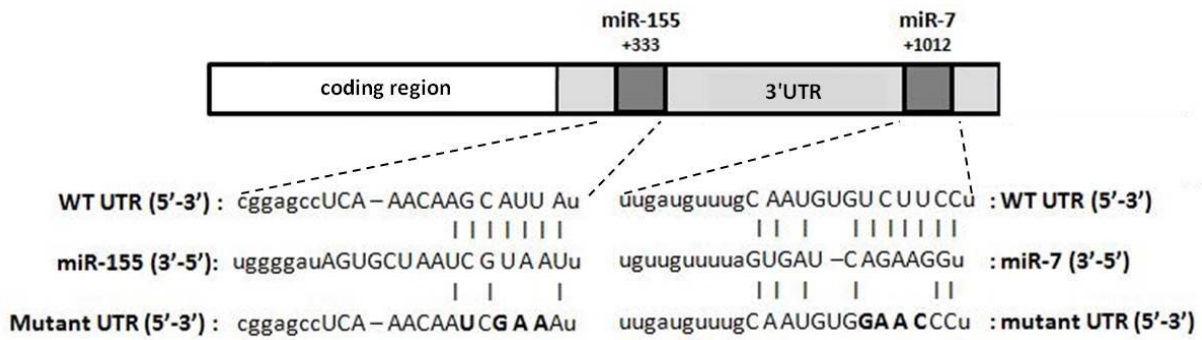
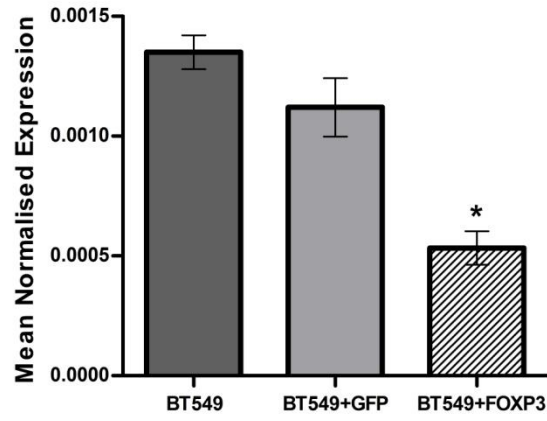


Figure 2 Endogenous SATB1 is reduced when *FOXP3* is over expressed in BT549

A)



B)

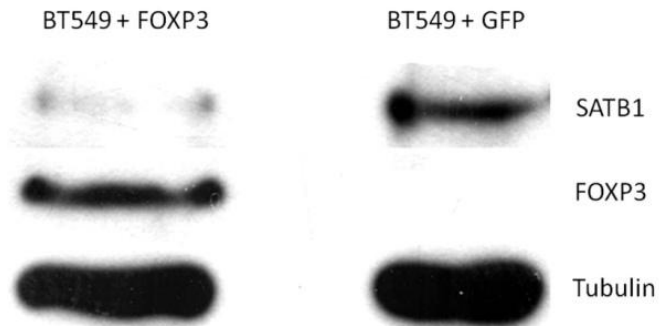




Figure 3 FOXP3 regulates the *SATB1* promoter

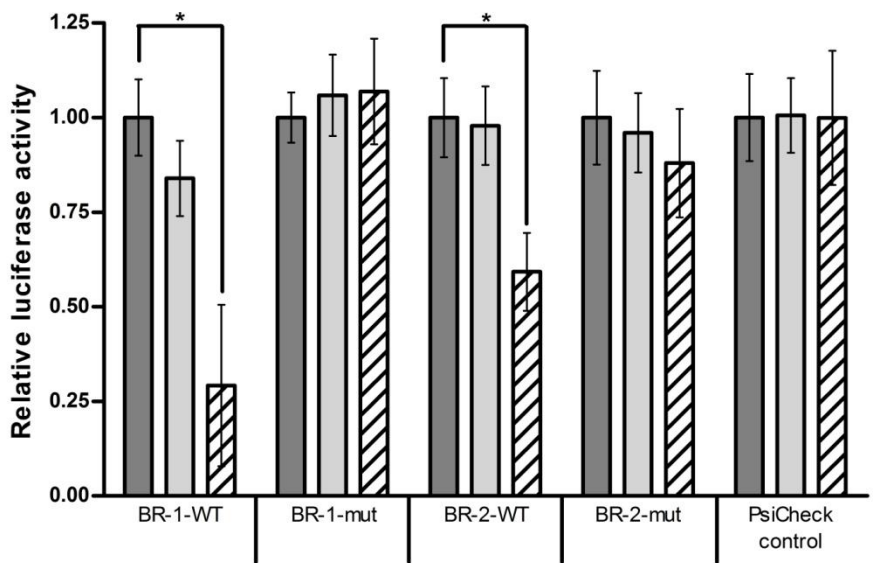


Figure 4 *FOXP3* over expression induces miRs -7 and -155 in BT549

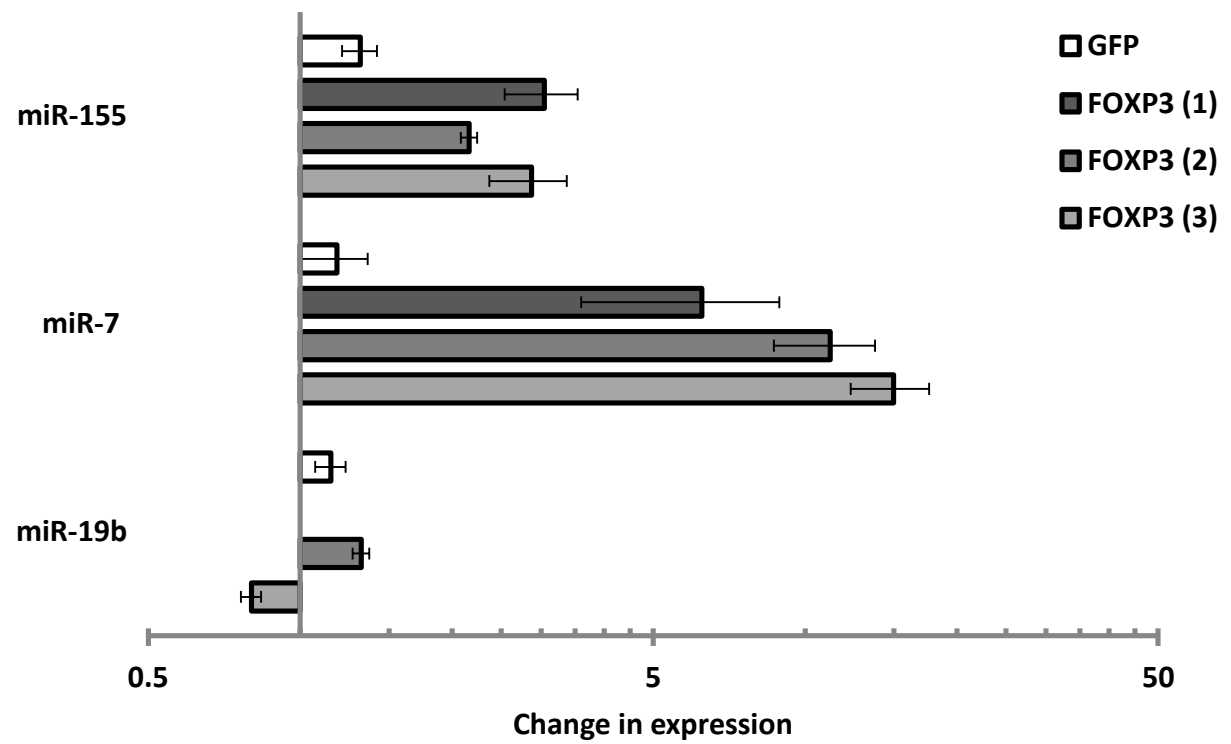
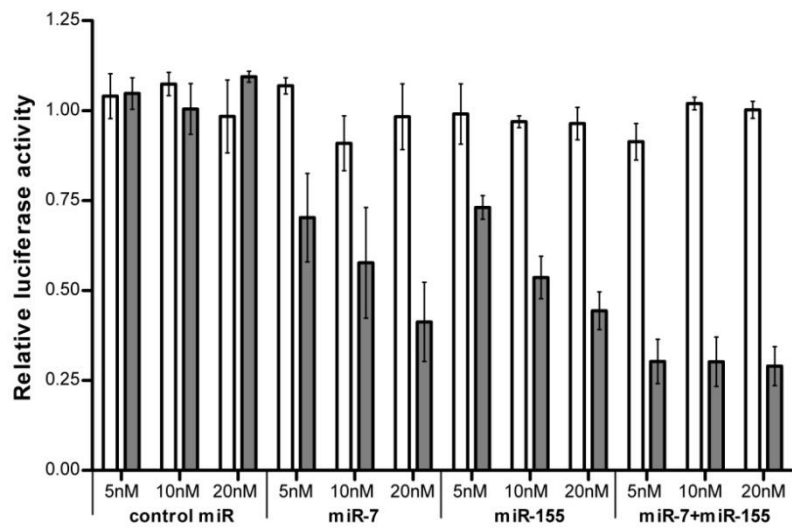


Figure 5 *SATB1* 3'UTR is directly targeted by miRs -7 and -155

A)



B)

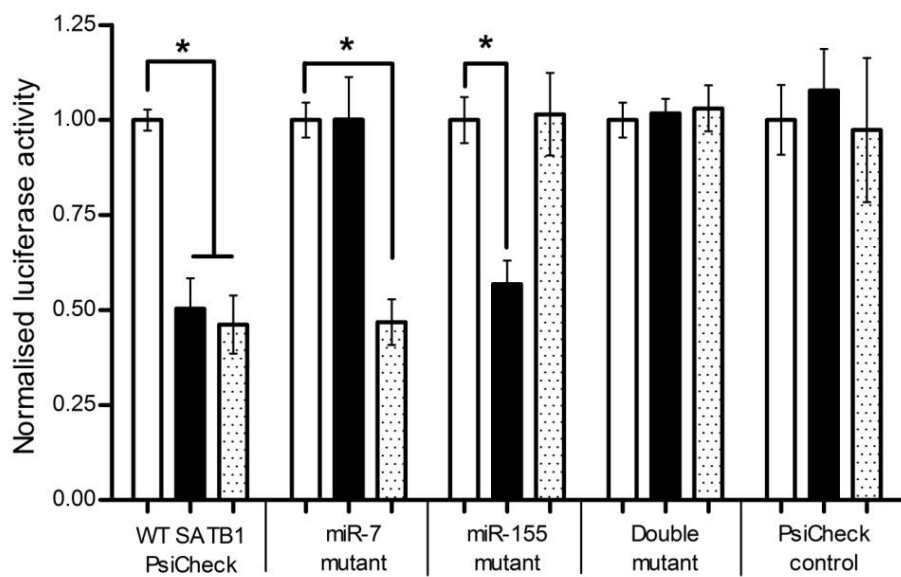


Figure 6 miR targeting of *SATB1* is blocked by PNA oligos

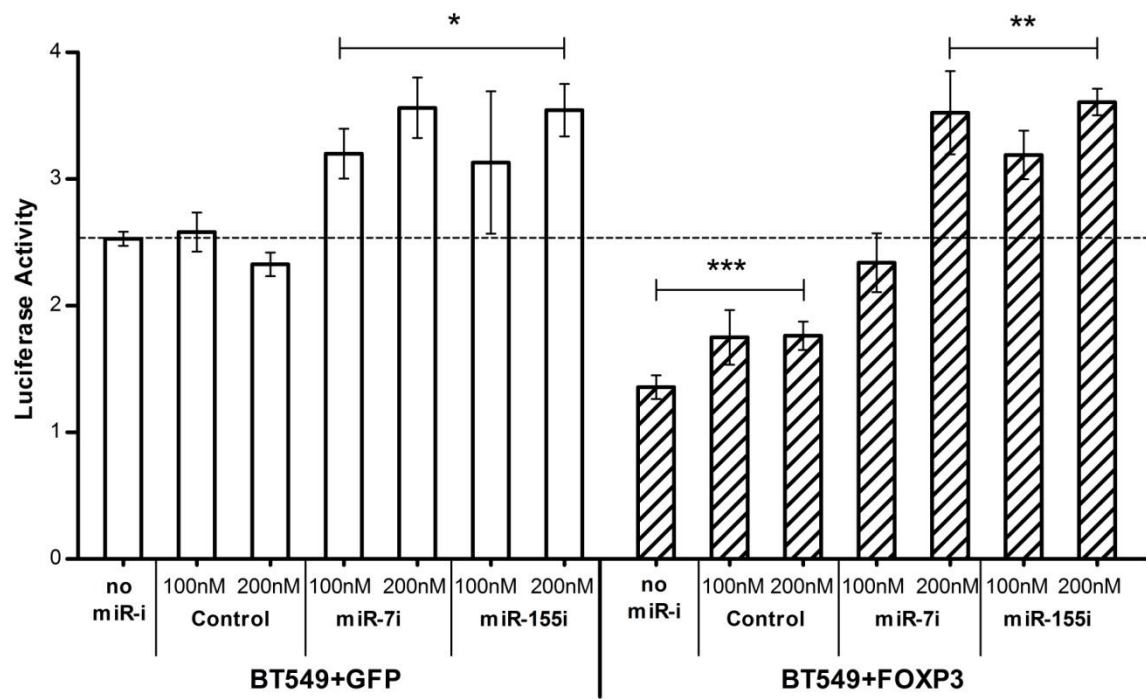
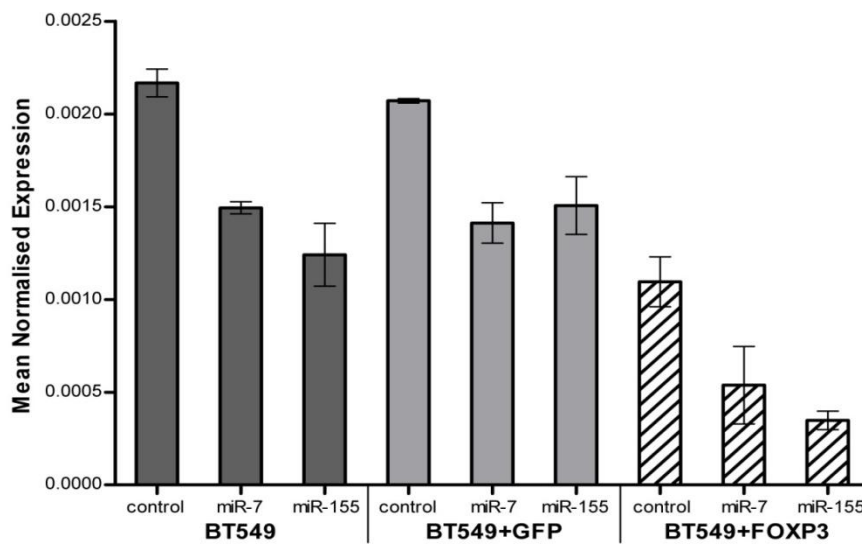
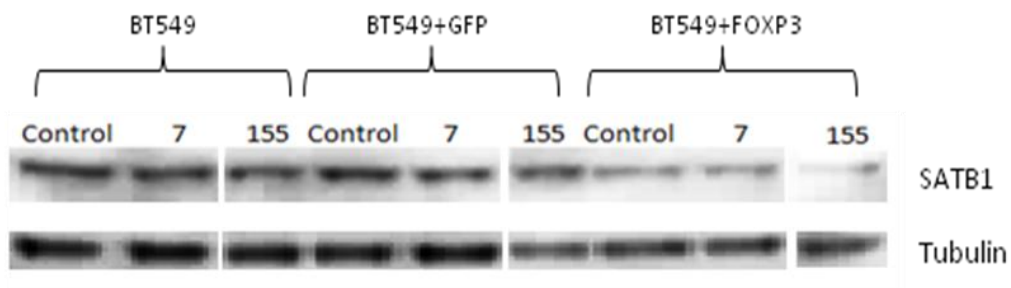


Figure 7 Endogenous SATB1 expression is reduced by miRs -7 and -155

A)



B)



C)

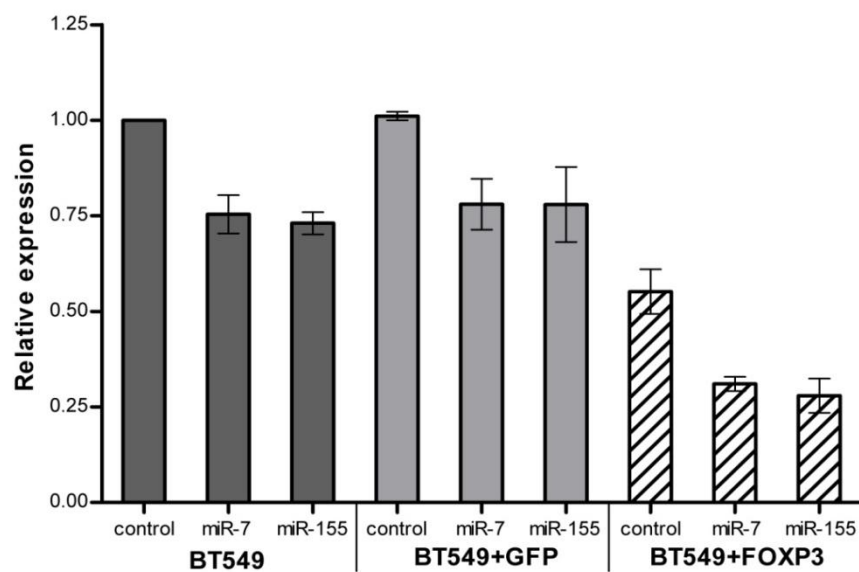
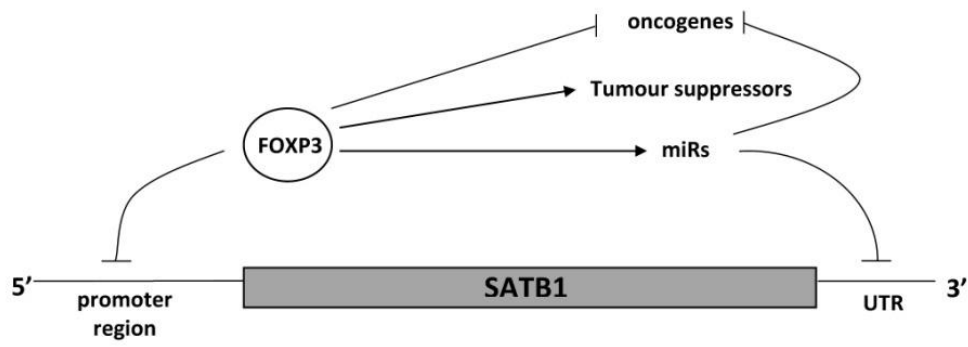


Figure 8 Model of feed-forward regulation of *SATB1* by FOXP3



## Figure Legends

### Figure 1

a) The gene structure of the human *SATB1* locus showing its 11 exons, and the human FOXP3 binding regions defined by chromatin immunoprecipitation in human regulatory T cells, revealing 5 potential FOXP3 binding regions (BR1-5). The *SATB1* proximal promoter is shown close to BR1 and BR2. b) Annotation of the *SATB1* 3'UTR showing predicted miR-155 and miR-7 seed sequences and subsequent introduced mutations.

### Figure 2

*SATB1* is repressed in BT549 cells over expressing *FOXP3*. a) *SATB1* mRNA levels in the parental BT549 cells (dark grey), BT549 over expressing GFP (light grey) and BT549 over expressing *FOXP3* (hatched) (n=3). b) SATB1 protein expression (upper row) as determined by western blot is detected in the parental BT549 cells, but is reduced in cells expressing *FOXP3* (middle row). The loading control is  $\alpha$ -tubulin (bottom row), \*= p<0.05.

### Figure 3

Luciferase reporter assay in BT549 cells using promoter constructs containing either WT FOXP3 binding sites at BR-1 and BR-2 or mutations of each site. When *FOXP3* is over expressed in these cells (hatched bars) the WT reporter is repressed, and repression is lost by mutation of either binding site. Data are expressed relative to the parental line (dark grey bars) and a GFP-transduced virus control (grey bars) is shown for n=3 transfections, \*= p<0.05.

### Figure 4

RT-PCR quantitation of miRs -7, -155 and -19b in three independent lentivirally transduced *FOXP3*-overexpressing pools of BT549 cells. Significant upregulation of miRs -7 and -155 is observed when *FOXP3* is expressed relative to levels in the control GFP-transduced cell line, with no change in miR-19b levels observed.

### Figure 5

a) Transient expression of pre-miRs -7 and -155 in BT549 cells expressing a luciferase reporter construct containing the *SATB1* 3'UTR (grey bars) or empty reporter vector (white bars), showing that either alone or in combination, these miRs repress the *SATB1* reporter in a dose dependant manner over the range 5-20nM. Results are normalised to the control pre-miR (n=3). b) Mutations of the miR-7 or miR-155 sites in the 3'UTR of the reporter vector were tested alone or in combination. Cells were transfected with control pre-miR (white bars), by pre-miR-7 (black bars) or pre-miR-155 (dotted bars) (n=3, \*= p<0.05).

### Figure 6

Transient expression of PNA inhibitor (PNAi) targeting miR-7 or miR-155 specifically inhibits the miR-mediated repression of the *SATB1* 3'UTR reporter construct in a dose dependant manner. BT549 cells expressing GFP (grey bars) or BT549 cells over expressing *FOXP3* (hatched bars) show a PNAi-induced increase in *SATB1* 3'UTR expression, (n=3, \*=

p<0.001 compared with the BT549 GFP no miR-i control, \*\*=p<0.001 compared to the BT546 FOXP3 no miR-i control, \*\*\*=p<0.05 compared to the BT549 GFP no miR-i control).

#### Figure 7

a) Expression of the endogenous *SATB1* mRNA is reduced when pre-miRs -7 or -155 are transiently expressed in BT549 cells. *SATB1* levels in the parental BT549 cells (dark grey bars) are reduced by the transfection of miR-7 or miR-155, as is seen in the GFP-transduced line (grey bars). Over expression of *FOXP3* alone (hatched bars) reduces *SATB1* levels, as shown by the control miR, and further reduces *SATB1* when the pre-miRs are transiently expressed in these cells. (Triplicate RNA analysis of n=3 transfection pools). b) A representative *SATB1* western blot of whole cell lysates from one of three independent transfection pools. c) Quantitation of *SATB1* protein levels by scanning densitometry of immunoblots, normalised to the tubulin loading control, n=3.

#### Figure 8

A schematic representation of the feed-forward regulation of *SATB1* by FOXP3. FOXP3 acts both directly at the 5' promoter region and indirectly by inducing miRs that target the 3'UTR. FOXP3 may induce other tumour suppressors and repress other oncogenes to give tight regulation of the protein.



## Supplementary Figures

Figure S1 The top disease associations of human FOXP3 ChIP targets identified with Ingenuity Pathway Analysis (IPA). Axis is Benjamini-Hochberg adjusted p-value.

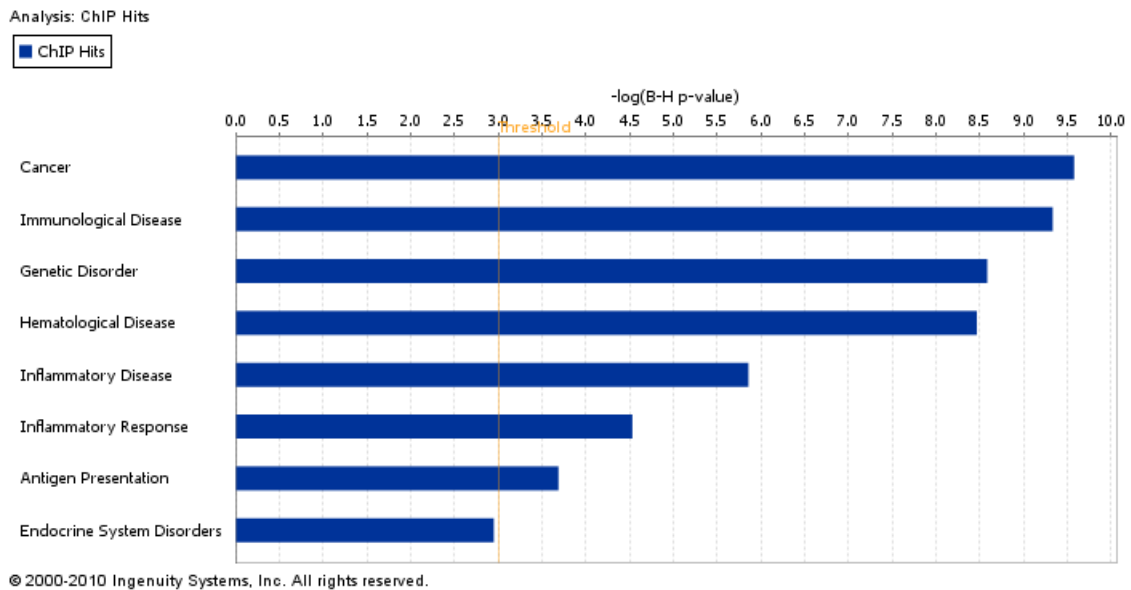


Figure S2 Stable expression of *FOXP3* in BT549 breast cancer cells.

The percentage of cells positive for GFP, expressed from an internal ribosome entry site in PLVEIG, was tracked for up to 8 passages post-sorting in *FOXP3* and control vector transduced cell lines by flow cytometry (n=3). Approximately 80% of BT549 cells transduced with the *FOXP3* expressing Lentivirus remained GFP positive for greater than 8 passages.

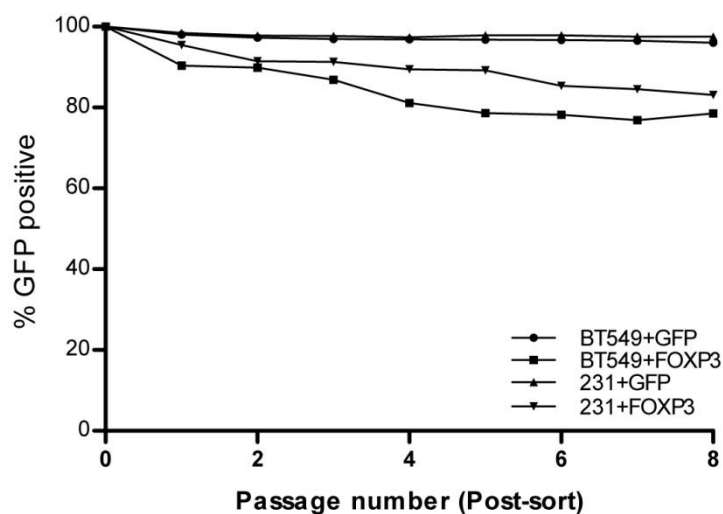


Figure S3 The proliferation of BT549 cells expressing *FOXP3* is impaired.

The proliferation of BT549 cells expressing *FOXP3* was compared to parental and control GFP expressing cells using the CellTiter 96 AQueous assay (Promega). Bars represent the SEM of triplicate samples. The figure is a representative of three experiments using cells derived from independent transductions.

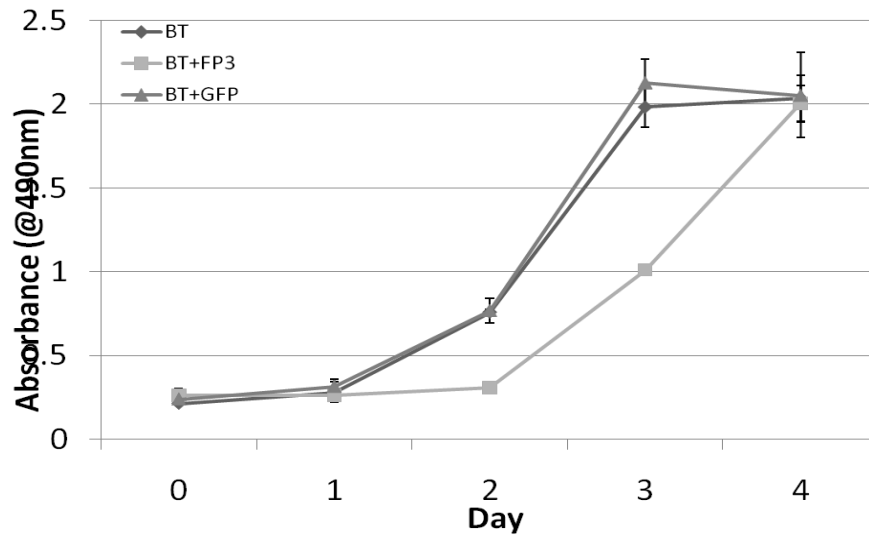


Figure S4. The *SATB1* 3'UTR is directly targeted by miR-7 and miR -155 in the MDA-MB-231 line

Transient expression of pre-miRs -7 and -155 in MDA-MB-231 cells expressing a luciferase reporter construct containing the *SATB1* 3'UTR (grey bars) or empty reporter vector (white bars), showing that either alone or in combination, these miRs repress the *SATB1* reporter in a dose dependant manner over the range 5-20nM. Results are normalised to the control pre-miR (n=3).

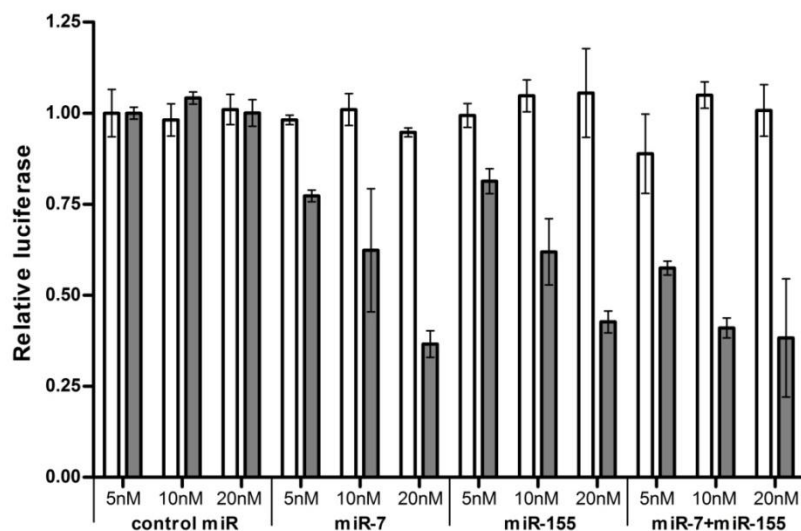


Figure S5. miR targeting of SATB1 is blocked in parental BT549 by PNA oligos.

Transient expression of PNA inhibitor (PNAi) targeting miR-7 or miR-155 specifically inhibits the miR-mediated repression of the *SATB1* 3'UTR reporter construct in a dose dependant manner. BT549 cells (white bars) show a PNAi-induced increase in *SATB1* 3'UTR expression, (n=3, \*= p<0.001 compared with the BT549 no miR-i control).

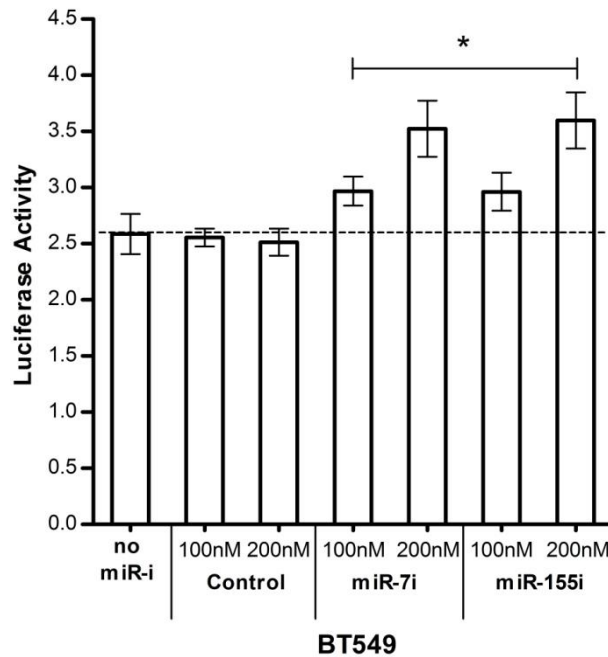


Figure S6. Validated miR-7 target are decreased in FOXP3 transduced cells.

The mRNA levels of the validated miR-7 targets, *EGFR*, *RAF-1* and *PAK-1*, were determined by qPCR in parental BT549 cells (dark grey), control GFP expressing (light grey) and *FOXP3* expressing (hatched) BT549 cells. A significant reduction in the mRNA level of these targets was observed in *FOXP3* expressing cells but not control GFP expressing cells. Shown is the data from three independent experiments.

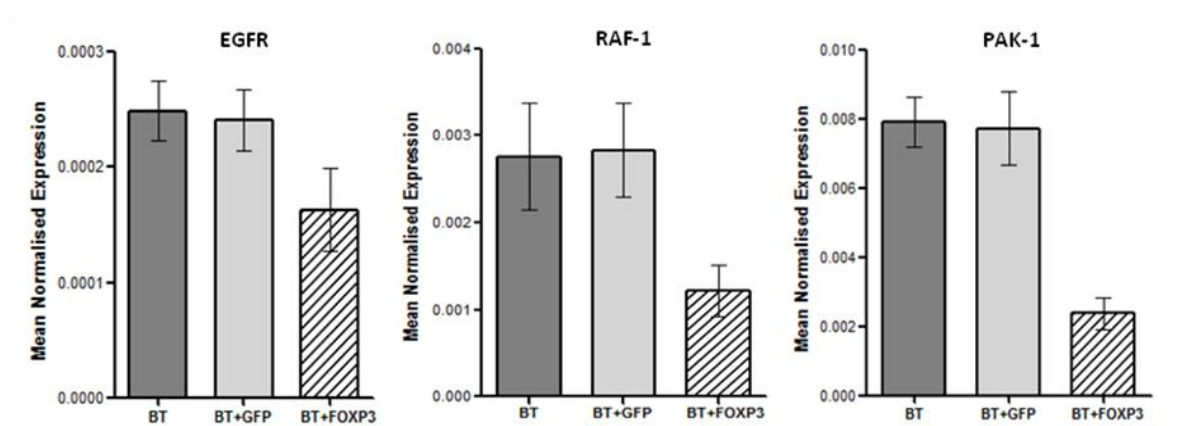


Table S1 List of PCR primers used in this study

	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>
<b>SATB1</b>	ACA GGT GCA AAA ATG CAG GGA	GCG TTT TCA TAA TGT TCC ACC AC
<b>RPL13a</b>	CGA GGT TGG CTG GAA GTA CC	CTT CTC GGC CTG TTT CCG TAG
<b>FOXP3</b>	GTC TGG GCT CAT AGG CAC ATT	ATG GCG TTC TGT GGA AGG C
<b>SATB1 3'UTR</b>	CAG CCA GCT GTA ACA AAA TAG C	GAA GAA GAG CTG TCA GTG GAA G
<b>miR-7 mutant</b>	TGT TTG CAA TGT GGA ACC CTT TGG TTT ACA	TGT AAA CCA AAG GGT TCC ACA TTG CAA ACA
<b>miR-155 mutant</b>	GAG CCT CAA ACA ATC GAA ATA CCT TCT GTG	CAC AGA AGG TAT TTC GAT TGT TTG AGG CTC
<b>RAF-1</b>	GCA CTG TAG CAC CAA AGT ACC	CTG GGA CTC CAC TAT CAC CAA TA
<b>PAK-1</b>	TAC CAG CAC TAT GAT TGG AGT CG	GGA TCG GTA AAA TCG GTC CTT CT
<b>EGFR</b>	GCG TTC GGC ACG GTG TAT AA	GGC TTT CGG AGA TGT TGC TTC

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