N-terminal PDZ-like domain of chromatin organizer SATB1 contributes towards its function as transcription regulator

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The special AT-rich DNA-binding protein 1 (SATB1) is a matrix attachment region (MAR)-binding protein that acts as a global repressor via recruitment of CtBP1:HDAC1-containing co-repressors to its binding targets. The N-terminal PSD95/Dlg-A/ZO-1 (PDZ)-like domain of SATB1 mediates interactions with several chromatin proteins. In the present study, we set out to address whether the PDZ-domain-mediated interactions of SATB1 are critical for its *in vivo* function as a global repressor. We reasoned that since the N-terminal PDZ-like domain (amino acid residues 1–204) lacks DNA binding activity, it would fail to recruit the interacting partners of SATB1 to its genomic binding sites and hence would not repress the SATB1-regulated genes. Indeed, *in vivo* MAR-linked luciferase reporter assay revealed that overexpression of the PDZ-like domain resulted in de-repression, indicating that the PDZ-like domain exerts a dominant negative effect on genes regulated by SATB1. Next, we developed a stable dominant negative model in human embryonic kidney (HEK) 293T cells that conditionally expressed the N-terminal 1–204 region harbouring the PDZ-like domain of SATB1. To monitor the effect of sequestration of the interaction partners on the global gene regulation by SATB1, transcripts from the induced and uninduced clones were subjected to gene expression profiling. Clustering of expression data revealed that 600 out of 19000 genes analysed were significantly upregulated upon overexpression of the PDZ-like domain. Induced genes were found to be involved in important signalling cascades and cellular functions. These studies clearly demonstrated the role of PDZ domain of SATB1 in global gene regulation presumably through its interaction with other cellular proteins.

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1. Introduction

Special AT-rich DNA-binding protein 1 (SATB1) acts as a global repressor of gene expression by recruiting co-repressor complexes to its binding sites (Yasui *et al.* 2002; Kumar *et al.* 2005, 2006; Purbey *et al.* 2009). Several functional domains confer unique functional properties to this chromatin organizer. The matrix attachment region (MAR) binding property of SATB1 is attributed to a DNA binding domain in its C-terminal half containing a Cut domain (CD) and a

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Abbreviations used: aa, amino acids; CD, Cut domain; CSBS, consensus SATB1-binding sequence; FN, fibronectin; HD, homeodomain; HEK, human embryonic kidney; HMM, hidden Markov model; MAR, matrix attachment region; NLS, nuclear localization sequence; NMTS, nuclear matrix targeting sequence; PDZ, PSD95/Dlg-A/ZO-1; SATB1, special AT-rich DNA-binding protein 1; SBS, SATB1 binding sites; Tet, tetracycline

homeodomain (HD) that together contribute towards recognition and high affinity binding of MARs (Dickinson *et al.* 1997; Purbey *et al.* 2008). Cleavage of SATB1 during T cell apoptosis at position 254 by caspase 6 causes detachment of SATB1 from the chromatin (Galande *et al.* 2001). This observation resulted in the identification of an oligomerization domain in the N-terminal of SATB1. Query using the 90–204 amino acids (aa) from the N-terminal region against the conserved domain database (version 1.01) classified it to be a homologue of PSD95/Dlg-A/ZO-1 (PDZ) domain. Hidden Markov model (HMM)-based alignment of the PDZ-like domain of SATB1 and several other PDZ domains indicated conserved residues at key positions (Galande *et al.* 2001). The consensus SATB1-binding sequence (CSBS) half-site is identical to the conserved element 'TAATA' typically bound by HDs. The high-affinity binding of SATB1 to DNA is dimerization-dependent (Purbey *et al.* 2008). Another region spanning aa 224 to 278 has been characterized as a nuclear matrix targeting sequence (NMTS) that is required for the transcription function of SATB1 (Seo *et al.* 2005). Furthermore, the region encompassing aa 20–40 was shown to be required for the nuclear localization of SATB1 and is conserved among the SATB family of proteins belonging to diverse species (Nakayama *et al.* 2005). Thus, SATB1 is a BUR-binding protein possessing multiple characterized functional domains, *viz.* the nuclear localization sequence (NLS), PDZ-like domain, nuclear matrix targeting sequence (NMTS), Cut-repeat-containing domain (CD) and homeodomain (HD).

PDZ domains are protein–protein recognition modules that play a central role in organizing diverse cell signalling assemblies. Most of the PDZ-containing proteins are associated with the plasma membrane and are involved in recruitment of signalling proteins to form a network of larger protein complexes at the membrane (Kim *et al.* 1995; Fanning and Anderson 1996; Gomperts 1996). The predominance of PDZ domains in metazoans indicates that this highly specialized scaffolding module probably evolved in response to the increased signalling needs of multicellular organisms (Harris and Lim 2001). Since SATB1 is the only chromatin-associated protein harbouring a PDZ-like dimerization domain, we hypothesized that it may act as the nuclear end-point of the PDZ-mediated signal transduction pathway that originates from the cell surface. Thus, SATB1 may act as a global regulator in response to the PDZ-mediated signal transduction cascade from cell surface to nucleus. The N-terminal region of SATB1 containing the PDZ-like domain has been shown to make complexes with HDAC1 and CtBP1 co-repressors, PCAF, CBP and p300 co-activator complexes (Kumar *et al.* 2005, 2006; Purbey *et al.* 2009) and also with β-catenin to mediate Wnt signalling (Notani *et al.* 2010). Specific residues within the PDZ-like domain are targets for phosphorylation and acetylation, which regulate the DNA binding activity of SATB1 and thus participate in multiple ways for the transcription of several target genes (Kumar *et al.* 2006). Thus, the PDZ-like domain of SATB1 not only serves as a dimerization domain indispensable to the DNA binding activity but also plays an important role in mediating protein–protein interactions required for the transcriptional function of SATB1. The N-terminal PDZ-like domain is therefore crucial for SATB1 function. We reasoned that upon overexpression, the PDZ-like domain exerts a dominant negative effect presumably by sequestering SATB1 interaction partners.

To test the effect of overexpression of the PDZ-like domain on the transcriptional activity of SATB1-regulated genes, we performed MAR-linked luciferase reporter assays and found that the PDZ-like domain exerts an effect opposite to that of SATB1. The role of SATB1 in gene regulation can be studied more effectively by establishing a stable conditional expression system wherein the expression of SATB1 can be controlled stringently by addition or removal of an inducer. Therefore, in the present study, in order to address the key question such as whether the PDZ-domain-mediated interactions of SATB1 are critical for its *in vivo* function as a global repressor, we developed a stable dominant negative model using HEK-293T cells that conditionally (Tet-on) express the PDZ-like domain of SATB1. Lack of MAR-binding domain does not allow it to bind to SATB1 binding sites, but simultaneously, it can potentially sequester the PDZ-interaction partners of SATB1. Induced and uninduced sets of dominant negative clones were subjected to microarray analysis with reference to the mock-transfected control. Gene expression profiling revealed interesting changes upon overexpression of the SATB1 PDZ-like domain as a dominant negative effector, yielding insight into the *in vivo* function of SATB1.

2. Materials and methods

2.1 *DNA constructs*

The 3XFlag-PDZ (1–204 aa) region was amplified from the 3XFlag-SATB1 construct (Kumar *et al.* 2006) using primers containing *Bam*HI and *Hin*dIII linkers. The resultant 650 bp PCR product was purified using PCR purification kit (Qiagen). The PCR product was digested and gel-purified using gel extraction kit (Qiagen) and was then ligated into *Bam*HI- and *Hin*dIII-digested pTER^{+} vector (gifted by Dr Hans Clevers). Positive clones were confirmed by restriction digestion and automated DNA sequencing.

2.2 *Luciferase reporter assay using treated cells*

The IgH-MAR-Luc- and IL2RαP-Luc-containing SATB1 binding sites (SBS) (Kumar *et al.* 2005, 2006) were used as reporter constructs. HEK-293T cells were seeded at

0.5×10^6 cells per well. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen). Cells were harvested 36 h post-transfection. Cells were harvested and resuspended in 100 µl of PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and 100 µl of luciferase substrate reagent (Perkin Elmer). Luciferase activity was measured using Top Count (Packard). The relative luciferase activity was plotted.

2.3 *Determination of optimum lethal dose of antibiotics for selection*

Lethal dose of blasticidine was optimized using 0.5, 1, 2, 3, 4, 5 and 6 µg/ml of blasticidine. Lethal dose of Zeocin was optimized using 50, 100, 200, 300, 400 and 500 µg/ml of Zeocin. Cell mortality was monitored over a period of 1 week under antibiotic selection pressure. Tet-repressor and operator constructs were co-transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen). Cells were treated with tetracycline (1.0 µg/ml) 24 h post-transfection and incubated for 12, 24 and 36 h.

2.4 *Stable co-transfection*

Tet-repressor expressing plasmid pCDNA6/TR (Invitrogen) and Tet-operator expressing $pTER^+$ 3XFLAG-PDZ plasmid construct were co-transfected into HEK-293T cells and were maintained in DMEM containing 10% FCS, 3 µg/ml blasticidine and 200 µg/ml Zeocin for 1 month until individual colonies (foci) appeared on culture plate. Individually 50 foci were picked and were expanded into separate dishes. Out of these, 30 optimally growing clones were grown further and then were induced with tetracycline.

2.5 *RNA isolation and RT-PCR*

Total RNA from uninduced and induced HEK-293T cells (4×10^6) was prepared using TRI reagent (Sigma). Imprompt II Reverse Transcriptase (Promega) was used for cDNA synthesis. Quantitative real-time PCRs were performed using Sybr green premix (Bio-Rad). Changes in threshold cycle (C_T) values were calculated as follows:

$$\Delta C_T = C_{T\ \text{Induced}} - C_{T\ \text{Uninduced}}$$

2.6 *Microarray analysis of gene expression in induced and uninduced cells*

Total RNA isolated from induced and uninduced clones was subjected to microarray analysis. Approximately 4–6 million cells were used in each case. Twenty µg of RNA was used for cDNA preparation. Amino allyl-dUTP (Sigma) was included in the RT reaction to allow for subsequent fluorescent labelling of cDNA using monofunctional NHS ester dyes (GE Healthcare) – experimental RNA samples were coupled to Cy5 and reference RNA samples were coupled to Cy3 NHS-ester dyes as described (*http://www.microarrays.org/protocols.html*). Labelled probes were then hybridized to 19K single spotted human cDNA microarrays (University Health Network Microarray Center, Ontario Cancer Institute, Canada) in 50% formamide, 5× SSC and 0.2% SDS, at 42°C overnight. After washing the slides at a maximum stringency of 0.2× SSC, 0.1% SDS, fluorescent images of hybridized microarrays were obtained using a ScanarrayExpress microarray scanner (Perkin Elmer). Primary data analysis was carried out using the Scanarray software (Perkin Elmer) and included filtration of bad spots. Spots with small diameters (<50 µm), low signal strengths (<350 fluorescence intensity units over local background in the more intense channel) and low regression ratios (<0.4) were discarded. Data was normalized using all good spots and exported to Microsoft Excel. Complete microarray datasets were analysed by unsupervised hierarchical clustering, using average linkage algorithm of 'Cluster'. The results of clustering were visualized by 'Tree View' using the software available at *http://www.microarrays.org/software.html*. Advanced analysis was also performed using the GeneSpring version 7.3 (Agilent) software. Two separate hybridizations were set, *viz.* induced *versus* untransfected and uninduced *versus* untransfected, and then were compared at each gene ID. Comparative analysis of both the hybridizations at each spot resulted in a cluster profile.

2.7 *Microarray validation*

Gene IDs were selected from the list of dysregulated genes and primers corresponding to these cDNAs were used to validate results of the microarray analysis. Changes in threshold cycle (C_T) values were calculated as follows:

$$\Delta C_T = C_{T\ \text{Induced}} - C_{T\ \text{Uninduced}}$$

Data are presented as fold change (fold change=$2^{-\Delta C_T}$).

3. Results and discussion

3.1 *N-terminal PDZ-like domain of SATB1 can act as dominant negative effector for transcription function of SATB1*

For direct assessment of the regulatory activity of PDZ-like domain on SATB1 function, luciferase reporter assay was performed. The heptameric IgH-MAR and the 555 bp promoter region of IL-2RαP, which are both known to

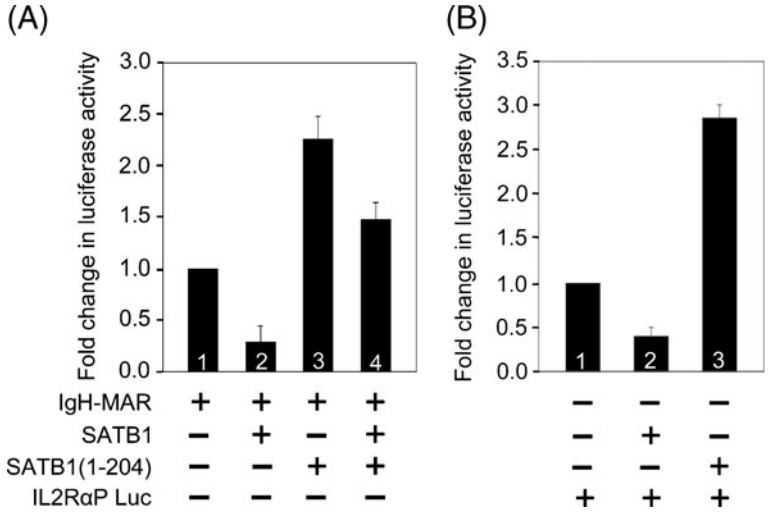

Figure 1. PDZ-like domain induces MAR-linked reporter gene expression. *In vivo* luciferase reporter assay was performed using the IgH-MAR-Luc (**A**) and IL-2RαP-Luc (**B**) reporter constructs as described in 'Materials and methods'. Briefly, 1 μg each of SATB1 and PDZ (1–204) constructs in p3X-Flag vector along with IgH MAR-luciferase reporter construct were transfected in the indicated combinations in HEK-293T cells. After 36 h, cells were harvested and luciferase activity was measured using the Luc-lite reagent. Luciferase activity is expressed as fold increase or decrease with respect to the control, which was set to 1. Relative luciferase units are represented as fold activity with respect to the reporter alone. Expression of the PDZ-like domain leads to derepression of MAR-linked reporter activity, as opposed to the repression observed by expression of SATB1. The statistical significance of differences between the treatment groups was calculated using the *t*-test, and the observed *p* values were always less than 0.05. Each error bar depicts the standard deviation calculated from triplicates.

contain strong SATB1-binding sites (SBS) (Kumar *et al.* 2005; Purbey *et al.* 2008), were used in conjunction with the promoterless luciferase reporter vector pGL3basic. SATB1 and its N-terminal (1–204) region were transiently overexpressed in HEK-293T cells. The N-terminal 1–204 aa region of SATB1 harbours both the nuclear localization signal (Nakayama *et al.* 2005) and the PDZ-like domain (Galande *et al.* 2001). We reasoned that since this domain (aa residues 1–

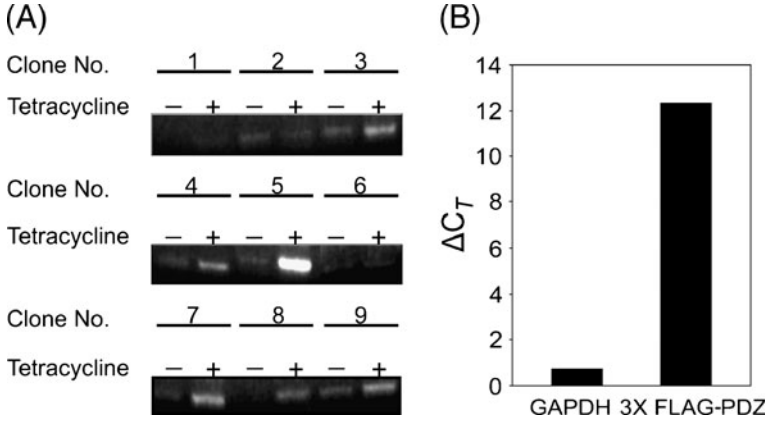

Figure 2. Selection and confirmation of the inducible stable clone. A stable dominant negative model in HEK-293T cells was developed which conditionally expresses the N-terminal 1–204 region harbouring the PDZ domain of SATB1. (**A**) RT-PCR analysis depicting induction upon tetracycline treatment. Addition of tetracycline induced expression of 3XFlag-PDZ in individual HEK-293T clones. RT-PCR was then performed to monitor the expression of 3XFlag-PDZ as described in 'Materials and methods'. (**B**) Among the clones checked, clone #5 showed the highest level of 3XFLAG-PDZ induction upon tetracycline treatment. Quantitative RT-PCR was performed, and the C_T value difference for the expression of 3XFLAG-PDZ between induced and uninduced samples indicated more than a 1000-fold induction. The C_T value difference for GAPDH internal control was only 0.7.

204) lacks DNA binding activity, it would fail to recruit the interacting partners of SATB1 to its genomic binding sites and hence it would not repress the SATB1-regulated genes. As expected, upon overexpression, SATB1 downregulated the luciferase activity (bar 2, figure 1A and B). Overexpression of the PDZ-like domain led to upregulation of SBS-linked reporter activity (bar 3, figure 1A and figure 1B) as opposed to the repression observed upon expression of SATB1. This could be attributed to the displacement of SATB1-bound repressor complexes by overexpressed PDZ-like domain. Further, when SATB1 and its PDZ-like domain were co-expressed in HEK-293T cells, we again observed a de-repression of MAR-linked luciferase activity (bar 4, figure 1A). The coexpression of SATB1 and its PDZ-like domain does not cancel their individual opposite effects. The de-repression in such case was lower than that observed with overexpression of PDZ alone, but nevertheless was significant and sufficient to confirm the dominant negative effect. Thus, these results demonstrated that the PDZ-like domain could act in a dominant negative fashion and completely abolish the transcriptional repressor function of SATB1.

3.2 *Generation of Tet-inducible SATB1 dominant negative cell line model*

We established a conditional expression system wherein the expression of PDZ is controlled stringently by the addition or removal of tetracycline (Tet), which is a better and more stringent method for studying the role of SATB1 in gene regulation. To generate a Tet -inducible expression system, the 3XFlag-PDZ was ligated into the cloning site of the

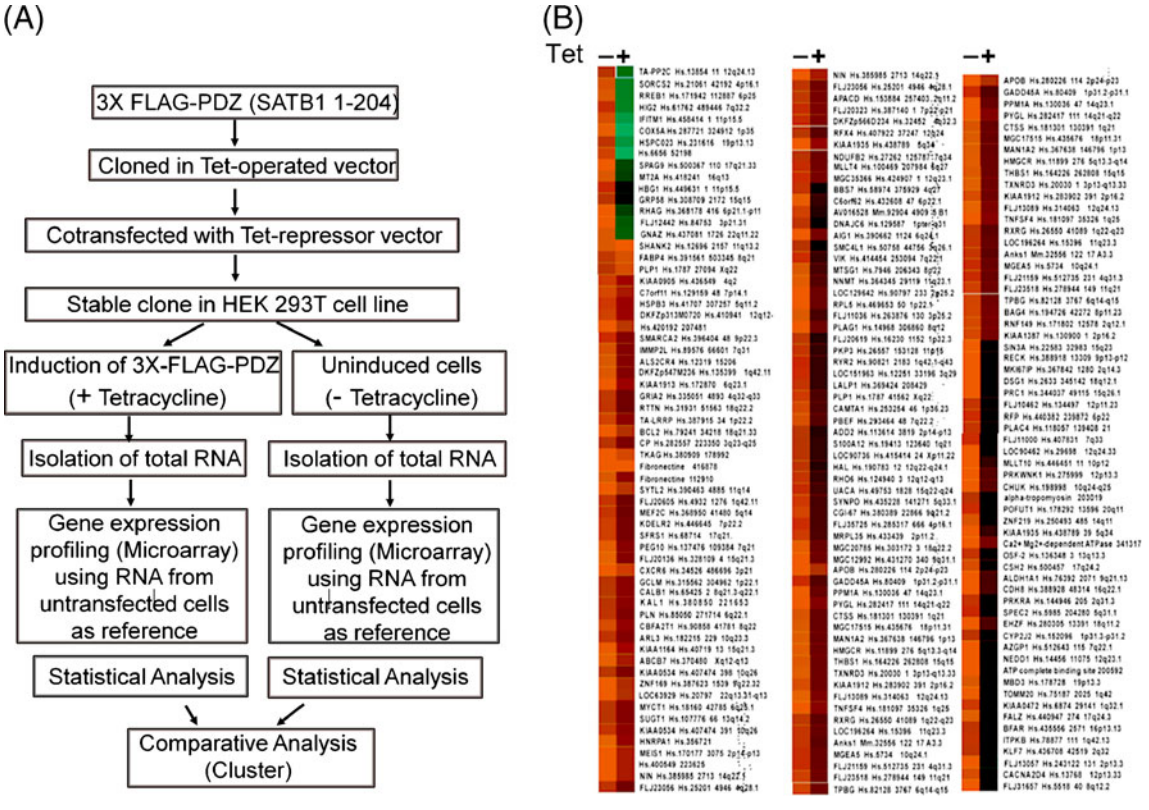

Figure 3. Gene expression profiling upon expression of dominant negative effector for SATB1. (**A**) Experimental design is depicted in form of a flowchart. The experiments were performed using HEK293T cells, which express SATB1 endogenously. Therefore, to account for this basal level of expression, microarray hybridizations were performed using uninduced *versus* untransfected and induced *versus* untransfected RNAs. RNA isolated from untransfected cells served as reference in both cases. (**B**) Clustering of gene expression profiles from Tet-induced and uninduced cells. Total RNA was isolated from induced and uninduced clone of SATB1-PDZ in HEK-293T cells and then was subjected to the microarray analysis using human 19 K cDNA chips as described in 'Materials and methods'. Two separate hybridizations were performed and two test conditions were used, *viz.* induced (treatment) *versus* untransfected and uninduced (treatment) *versus* untransfected, and were then compared at each gene ID using 'GenePix Pro'software. Comparative analysis of both the hybridizations at each spot resulted in a cluster profile. Only the dysregulated genes are shown. Green indicates downregulated genes and red indicates upregulated genes.

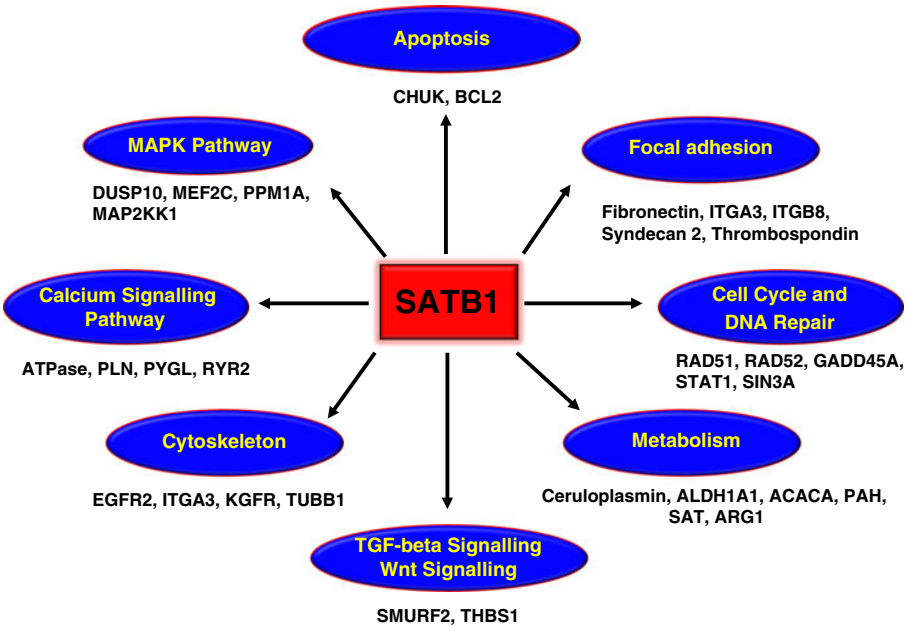

Figure 4. SATB1 regulates genes involved in key biological pathways. Overexpression of the PDZ-like domain resulted in dysregulation of over 600 genes out of 19000. Genes that were significantly dysregulated were analysed further using 'Pathways' and were found to be involved in different dynamic biological pathways as depicted above. Pathway analysis of gene expression profiling was performed as described in 'Materials and methods'. Only the most significantly affected pathways and their key genes are depicted.

pTER$^+$ vector (Van de Wetering *et al.* 2003). The resulting construct was transfected into the HEK-293T cells and was selected on blasticidine-containing media. Clones were expanded and re-transfected with pCDNA6/TR-containing Zeocin as selection marker (figure 2), enabling double selection. The double stable clone was selected in media containing both blasticidine and Zeocin. In presence of Tetracycline, the Tet repressor cannot bind the operator. Therefore, genes cloned in pTER$^+$, are expressed under very stringent control.

Eighty clones were screened for expression and induction levels of 3XFlag-PDZ. All the clones were induced with media containing Tetracycline at a final concentration of 1 mg/ml. Uninduced cells grown in absence of tetracycline

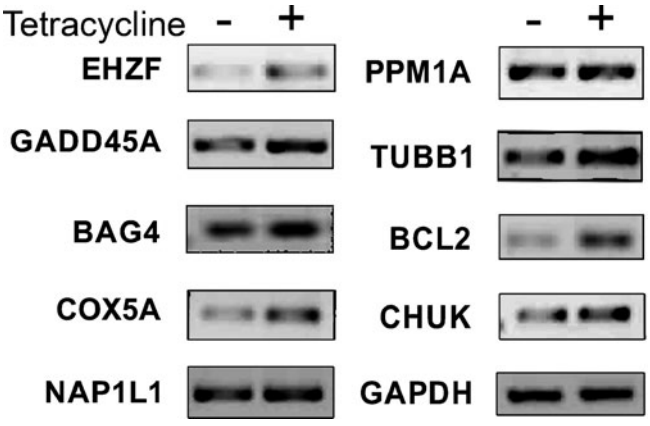

Figure 5. Validation of gene expression profiling data. From gene expression profiling data, a few genes were selected from the list of significantly (fold change >2) dysregulated genes and were then validated further. Semiquantitative RT-PCR analysis of a few previously established and new SATB1 targets was performed as described in 'Materials and methods'. The panels show the level of different transcripts before and after induction. Gene names are indicated on the left of each panel.

Table 1. Validation of gene expression profiling data

Genes		Fold difference in expression	
Gene	Gene ID	Microarray	Real-time RT-PCR
FN	2335	12.0	10.8
BAG4	9350	2.26	2.0
GADD45A	1647	2.14	2.0
NAP1L1	4673	1.96	2.5
COX5A	12858	3.96	4.0
PPMA1	9550	2.74	1.74
TUBB1	81027	2.08	2.0
BCL2	570772	3.32	3.5
EHZF	25925	5.35	12.0
CHUK	1147	1.93	2.0

The table shows comparison between fold change values obtained from quantitative RT-PCR and microarray analysis of few selected genes. Gene names are indicated on the left of each row and corresponding gene IDs are provided.

were used as control. Total RNA was isolated from the uninduced and induced clones. RT-PCR was performed using primers specific for 3XFlag-PDZ. The screened clones showed varying levels of 3XFLAG-PDZ induction (figure 2A). The clone that exhibited highest level of 3XFLAG-PDZ induction was subjected to quantitative PCR analysis (figure 2B). This clone yielded over 1600-fold induction of the 3XFlag-PDZ and was therefore chosen for the microarray-based gene expression profiling.

3.3 *Overexpression of PDZ-like domain leads to upregulation of multiple genes*

SATB1 is known to regulate the transcription of large number of genes (Kumar *et al.* 2006; Han *et al.* 2008; Purbey *et al.* 2009). The N-terminal PDZ-like domain of SATB1 lacks DNA binding activity (Purbey *et al.* 2008) and therefore affects transcription indirectly. Such effects are better tested across large number of genes simultaneously. We therefore adopted the microarray profiling approach to analyse the genes dysregulated upon conditional overexpression of the PDZ-like domain of SATB1 in HEK-293T cells. For this, we set two test conditions, *viz.* induced (treatment) *versus* untransfected and uninduced (treatment) *versus* untransfected cells (depicted as a flowchart in figure 3A). This strategy of using two test conditions was extremely important for the outcome of the study. The labelled cDNAs prepared from RNA isolated from 3XFlag-PDZ stably expressing HEK-293T cells and control untransfected cells were used to hybridize 19K human cDNA array. The fold differences in

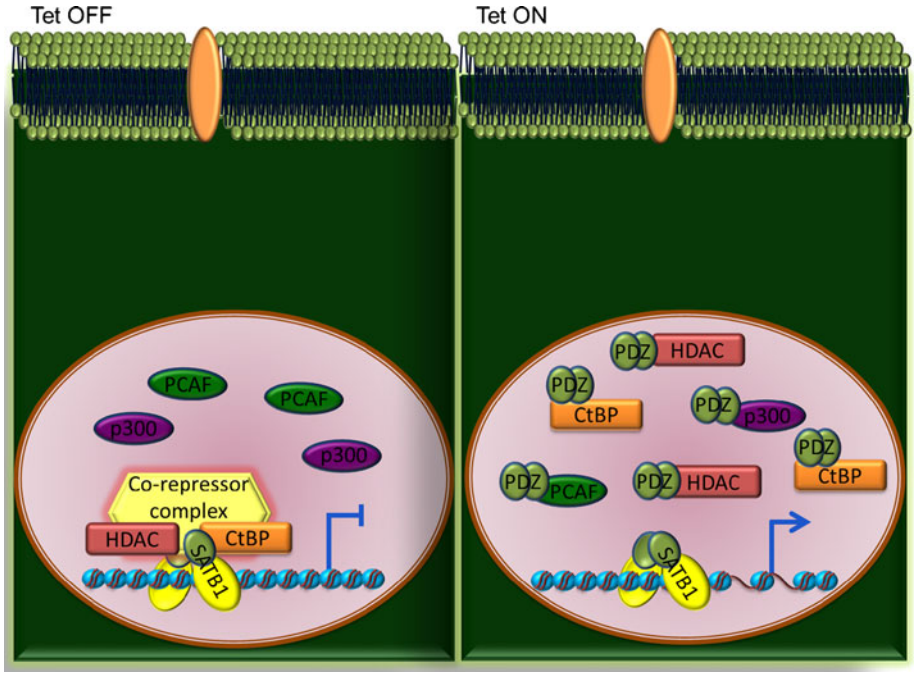

Figure 6. Schematic model depicting the dominant negative function of PDZ-like domain. SATB1 recruits the CtBP1:HDAC1-containing repressor complexes via its PDZ-like domain and downregulates transcription of its targets. Upon overexpression of the PDZ-like domain, cofactors specifically bind to the abundant PDZ molecules, which do not have DNA binding activity. This leads to the sequestration of various cofactors bound to SATB1, thus leading to dysregulation of genes mostly in form of derepression.

expression of representative genes showed distinct profiles. Genes that were downregulated in the absence of Tet were dramatically upregulated upon Tet induction. Clustering of microarray data revealed that 600 out of 19000 genes analysed were significantly upregulated upon induction of expression of the PDZ-like domain by Tet (figure 3B). Pathway analysis of genes upregulated upon Tet induction revealed that these genes were involved in various functions, *viz.* multiple signalling cascades including Wnt, Notch and TGF-β, extracellular attachment, cellular integrity and structure (figure 4). The gene expression profile unequivocally proved the role of SATB1's PDZ-like domain in global gene regulation presumably through its interaction with other chromatin proteins.

Next, we revalidated results of the gene expression profiling by quantitative RT-PCR analysis. Towards this end, we picked 10 genes from the list of significantly (fold change >2) dysregulated genes and validated by RT-PCR (figure 5) and by real-time RT-PCR under induced and uninduced conditions (table 1). GAPDH was used as an internal control. Known SATB1 targets such as *COX5A*, *PPM1A*, *BCL2* and *CHUK* (Notani *et al.* 2010) as well as genes not known to be regulated by SATB1 such as Fibronectin (*FN*), *BAG4*, *EHZF*, *TUBB1* and *GADD45A* were significantly upregulated upon overexpression of the PDZ-like domain (table 1), indicating that SATB1 might be involved in their negative regulation. Since the fold changes derived from quantitative RT-PCR analysis and microarray analysis are comparable, the results of gene expression profiling are validated. These results unequivocally establish that SATB1 downregulates gene expression at global level. This approach unraveled a set of newly identified SATB1 target genes involved in multiple signalling pathways. Taken together, these studies demonstrate the role of the PDZ-like domain of SATB1 in global gene regulation presumably through its interaction with other chromatin proteins (figure 6).

To date, *in vivo* studies on gene regulation mediated by SATB1 have been restricted to SATB1-null mice (Alvarez *et al.* 2000). However, because of the severe defects in T cell development in these mice, it is not possible to analyse the precise function of SATB1 in T cell development and gene regulation. Even in a cell line model, constitutive depletion or overexpression of SATB1 results in apoptotic cell death (Galande *et al.* unpublished observations). SATB1 has two main functional domains: N-terminal PDZ-like signalling domain and C-terminal DNA binding domain. Overexpression of the C-terminal half containing the DNA binding domain has no effect because the N-terminal half which confers the dimerization ability is indispensible for the DNA binding activity of SATB1 (Purbey *et al.* 2008).

In summary, this study identified several new SATB1 targets that might potentially undergo change in expression upon modifications of the PDZ-like domain. Furthermore, this study also raises an important possibility that various signalling pathways such as the Wnt signalling could, in turn, induce these targets. SATB1 mediates Wnt signalling by recruitment of β-catenin via the PDZ-like domain to its targets (Notani *et al.* 2010). Elucidation of the mechanism of how the repressor complexes associated with the global transcription factors such as SATB1 are replaced by activator complexes would provide a complete understanding of the mechanism of transcriptional activation following a signalling process. In all such situations, associations of the PDZ-like domain with various chromatin modifiers that are in turn dictated by the post-translational modifications of SATB1 at this domain might play a decisive role. Hence, the key question that remains to be answered is whether SATB1-PDZ exerts its effect by displacing or sequestering the interacting partners of SATB1 from it, resulting in dysregulation of SATB1-targeted genes (figure 6). The dominant negative effect imparted by the overexpression model described here is potentially useful in an *in vivo* scenario such as a transgenic animal model in which its effect can be monitored with respect to a biological phenomenon such as development and/or differentiation of cells.

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