

**SATB1 tethers multiple gene loci to reprogram expression profile driving
breast cancer metastasis**

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ABSTRACT

Global changes in gene expression occur during tumor progression, as indicated by expression profiling of metastatic tumors. How this occurs is poorly understood. SATB1 functions as a genome organizer by folding chromatin via tethering multiple genomic loci and recruiting chromatin remodeling enzymes to regulate chromatin structure and expression of a large number of genes. Here we show that SATB1 is expressed at high levels in aggressive breast cancer cells, and is undetectable in non-malignant breast epithelial cells. Importantly, RNAi-mediated removal of SATB1 from highly-aggressive MDA-MB-231 cells altered the expression levels of over 1200 genes, restored breast-like acinar polarity in three-dimensional cultures, and prevented the metastatic phenotype *in vivo*. Conversely, overexpression of SATB1 in the less-aggressive breast cancer cell line Hs578T altered the gene expression profile and increased metastasis dramatically *in vivo*. Thus, SATB1 is a global regulator of gene expression in breast cancer cells, directly regulating crucial metastasis-associated genes, including *ERRB2 (HER2/NEU)*, *TGF- β 1*, *matrix metalloproteinase 3*, and *metastasin*. The identification of SATB1 as a protein that re-programs chromatin organization and transcription profiles to promote breast cancer metastasis suggests a new model for metastasis and may provide means of therapeutic intervention.

Introduction

Metastasis is a final step in solid tumor progression and is the most common cause of death in cancer patients¹. Metastasis is believed to occur through a multi-step process, beginning with invasion of the tumor cells into the adjacent tissues, intravasation, which represents entry of tumor cells in the systemic circulation, survival in circulation, extravasation to distant organs, and finally growth of cancer cells in a new host environment to produce secondary tumors^{2,3}. The mechanisms by which tumor cells acquire metastatic functions are largely unknown.

Gene expression profiling of human breast carcinomas have identified characteristic gene expression patterns often associated with poor prognoses⁴⁻⁶. The presence of such poor-prognosis gene signatures, detectable even in primary tumors, challenges the long-held view that metastatic cells are rare and evolve during late stages of tumor progression via a series of genetic changes. Indeed there is little mechanistic evidence for how poor prognosis-associated gene expression profiles arise. Such expression patterns may arise fortuitously in some cells in primary tumors, or a functional mediator may be newly expressed that specifically directs changes in the expression pattern of the primary tumor cells, resulting in a metastatic phenotype.

To search for a key factor that may change the global gene expression pattern to favor metastasis, we focused on SATB1, a protein we had described previously to function as a ‘genome organizer’. SATB1 has a ‘cage-like’ protein distribution surrounding heterochromatin, folds chromatin by tethering specialized DNA sequences and serves as a landing platform for the assembly of chromatin remodeling/modifying complexes with the anchored genomic loci, thereby regulating expression of a large body of genes. Thus, SATB1 constitutes a functional nuclear architecture, referred to as ‘the SATB1 network’, since it establishes a regulatory network of gene expression⁷⁻¹⁰. Although SATB1 function has been studied mostly in the T cell lineage, if SATB1 were expressed in breast cancer cells, it would be expected to exert a global gene regulatory activity in these cells as well. SATB1 tethers chromatin by binding to specialized DNA sequences called base unpairing regions (BURs), which have a unique physical property in which DNA bases become extensively unpaired under negative superhelical strain¹¹⁻¹³. This property is due to a specialized DNA context (an ATC sequence context) characterized by a cluster of sequence stretches with well mixed As and Ts but either Cs or Gs exclusively on one strand^{7,11}. BURs are found in intergenic and intronic sequences, and are enriched near

regulatory regions, but absent in the coding regions of genes. SATB1 binds *in vivo* to these regions of DNA and recruits chromatin modifying factors, such as ATP-dependent chromatin remodeling enzymes and histone modification enzymes, to determine region-specific epigenetic statuses and chromatin conformation^{9,10}.

Gene targeting experiments revealed that SATB1 regulates the expression of hundreds of genes during T-cell differentiation⁸. T cell development is arrested at a premature stage in SATB1-null mice, resulting in accumulation of CD4⁺/CD8⁺ cells. We have shown recently that upon T-helper 2 cell activation, SATB1 is induced rapidly and binds at multiple sites in the cytokine gene cluster region, where it mediates the formation of a three-dimensional chromatin architecture required for upregulation of multiple cytokine genes¹⁴. Therefore, as an organizer of chromatin structure, SATB1 is an important regulator of a network of gene expression that determines T cell specificity, development and function.

Because of its function in regulating chromatin organization and expression of a body of genes, we investigated whether SATB1 might be involved in the global changes in gene expression that occur during malignant progression. We show here that in breast cancer cells, SATB1 is expressed primarily in aggressive breast cancer cells, but is undetectable in non-cancerous breast epithelial cells. In fact, SATB1 regulates the expression of a select set of genes that promote the metastatic phenotype and the metastatic activity of breast cancer cells increases as SATB1 levels are increased. Conversely, the metastatic phenotype of aggressive breast cancer can be reverted to non-aggressive phenotypes when SATB1 is down modulated.

RESULTS

SATB1 is expressed in aggressive breast cancer cells

We examined a number of breast epithelial cell lines for expression of SATB1; these included normal human mammary epithelial cells (HMEC) and their immortalized derivatives, non-metastatic cancer cell lines and metastatic cell lines. We detected SATB1 expression in only a subset associated with advanced-stage or metastatic cancer, and not in phenotypically normal HMECs or immortalized non-cancerous cells (Fig 1a).

We also examined SATB1 expression levels in 28 human primary breast tumor samples, including moderately or poorly differentiated ductal carcinomas, and 10 adjacent tissues as controls. The pathological analyses for these tumor samples were made prior to our SATB1

expression analysis. We detected high levels of SATB1 expression in all lymph-node positive, poorly differentiated infiltrating ductal carcinomas, and low-level expression in some, but not all, moderately differentiated tumor samples (Fig. 1b and Supplemental Table 1). SATB1 protein was detected in 23 of the 28 tumor samples examined. Among the 28 tumor samples, sixteen were metastatic breast carcinomas, and SATB1 was expressed in all of them with very high statistical significance ($P < 0.0001$) compared to either moderately differentiated tumor or normal tissue samples. SATB1 was not detected in any normal adjacent tissues (Fig. 1b).

Immunohistochemical analysis revealed that SATB1 expression was restricted to cells in cancerous areas of tissue samples, primarily in regions of highly disorganized morphology (Fig. 1c). SATB1 expression could be detected in aggressive breast carcinoma regardless of their classification ‘category’ based on available marker expression (Supplemental Table 1).

SATB1 is required for invasive activity and anchorage-independent cell growth

We used RNA interference (RNAi)¹⁵ to determine whether SATB1 is required for the invasive and metastatic phenotypes of breast cancer cells. The highly-metastatic MDA-MB-231 cell line, derived from the pleural effusion of a breast cancer patient who developed widespread metastases years after removal of her primary tumor¹⁶, expressed high levels of SATB1. Expressing short hairpin-interfering RNAs (shRNA) targeted against two SATB1 sequences in this cell line reduced its expression dramatically. Expression of SATB1 was lowered by 70% and 90%, respectively, in two transduced cell lines, which we named SATB1-shRNA1 MDA and SATB1-shRNA2 MDA cells (Fig. 2a). SATB1 expression remained unaltered in MDA-MB-231 cells expressing an shRNA whose sequence did not match any known human gene (control cells).

We examined whether loss of SATB1 expression affects cancer cell proliferation by culturing SATB1-shRNA1 MDA and SATB1-shRNA2 MDA cells on plastic dish {two-dimensional (2D) culture} or on a reconstituted basement membrane derived from Engelbreth-Holm-Swarm Tumor (MatrigelTM) {three-dimensional (3D) culture}. The proliferation rates of SATB1-shRNA1 and SATB1-shRNA2 MDA cells were significantly reduced in both 2D and 3D cultures up to 11 days in culture, compared with their parental cell line and control cells (Fig. 2b). Furthermore, the invasive capacity *in vitro* of the SATB1 knock-down cells was reduced by 80–85% (Fig. 2c). Consistent with a reduction in invasiveness, depletion of

SATB1 prevented colony formation of these cell lines in soft agar, indicating restoration of their anchorage-dependent growth (Fig. 2d).

SATB1 depletion restores non-cancerous morphology for MDA-MB-231 cells

Mammary epithelial tumors *in vivo* typically exhibit a disruption of tissue organization and polarity. Because depletion of SATB1 reduced the aggressive behavior of MDA-MB-231 cells in *in vitro* assays, we examined whether cell morphology was also affected. In 2D cultures, immunolocalization of β -catenin in control cells revealed the absence of defined cell-to-cell junctions (Fig. 3a). Depletion of SATB1 expression, however, restored proper cell-to-cell junction formation. SATB1-shRNA1 MDA cells also grew as monolayer, unlike its parent cancer cell line.

In 3D cultures, SATB1-shRNA1 MDA cells maintained a spherical structure, referred to as cobble-stone like morphology, for up to 10 days in culture. In contrast, the control cells displayed a stellar-like scattered pattern characteristic of aggressive breast cancer cells. Therefore, loss of SATB1 expression caused the cancer cells to regain the non-aggressive cell morphology. We also found that SATB1 depletion caused a similar reversion to a non-aggressive phenotype in the highly invasive BT549 breast tumor cell line and reduction of invasive activity *in vitro* (Supplemental Fig. 1a-d). When non-transformed mammary epithelial cells are cultured on 3D, glandular-like structures (acini) are formed with a hollow lumen surrounded by polarized epithelial cells¹⁷⁻²⁰. We tested whether SATB1 depletion in MDA-MB-231 cells restores the acinar structures. In control MDA-MB-231 cells, immunofluorescence analysis using markers of normal acinar formation such as filamentous actin (F-actin), β -catenin and the basal ECM receptor, integrin α 6, revealed a disorganized structure (Fig. 3b). In contrast, the normal acinar structures were restored to SATB1-shRNA1 MDA cells in 3D culture, as revealed by uniform and polarized nuclei, cortically organized F-actin, β -catenin localized at the lateral cell-cell junctions, and α 6 integrin polarized to the basal surface of cells. Similar results were obtained with multiple other MDA-MB-231 clones in which SATB1 was confirmed to be down-regulated more than 70% by SATB1-shRNA expression. None of these features, indicating basal polarity, were present in the control MDA-MB-231 cells (Fig. 3b). Therefore, SATB1 knock-down in MDA-MB-231 breast

cancer cells causes them to acquire polarized cellular structures similar to that of normal mammary epithelial cells.

We next examined the nuclear distribution of SATB1 in aggressive breast cancer cell lines. Immunostaining of breast cancer cells grown in 2D cultures revealed that SATB1 is randomly distributed into small foci throughout the nucleus (punctate pattern), but is not present in regions of heterochromatin. However, SATB1 showed a dramatic redistribution when cells were switched to 3D cultures, with the small foci growing larger and eventually connecting into a network-like structure, surrounding heterochromatin (Fig. 3c). This expression pattern is to some extent reminiscent of SATB1 nuclear distribution in thymocytes¹⁰ and activated T cells¹⁴, in which the network-like structure serves as an anchorage platform for genomic DNA to fold chromatin to regulate region-specific epigenetic modification and gene regulation.

SATB1 is required for metastasis of MDA-MB-231 cells to lung

Because SATB1 knock-down reduces breast cancer cell invasion and colony formation in soft agar and restores normal cell morphology *in vitro*, we evaluated its *in vivo* effects on metastasis. SATB1-shRNA1 MDA, SATB1-shRNA2 MDA, and control cells (1×10^6 cells) were injected intravenously into the lateral tail vein of 6-week-old athymic mice to evaluate metastasis of these cancer cell lines to lung. In mice, the metastasis of orthotopically grown tumors derived from human MDA-MB-231 cells is a relatively rare event. Therefore, by directly introducing cells into the circulation, we examined the requirement of SATB1 in cancer cell survival in circulation and extravasation to and growth in the lung.

By nine weeks after tumor-cell injection, the lungs of mice injected with the control cells had formed numerous nodules, ranging in number from 125 to 160 per lung in all six mice analyzed (Fig. 4a,b). In contrast, the number of lung metastases was greatly reduced in mice injected with the SATB1 knock-down cells, SATB1-shRNA1 MDA cells, ranging from 0 to only 50 per lung among six mice. The lung metastases derived from the SATB1-depleted tumor cells were also much smaller in size than those derived from the control cells. Lung metastases from the second knock-down cell line, SATB1-shRNA2 MDA cells, were not observed in five out of six mice, and one mouse injected with these cells developed only five nodules/lung (Fig. 4b). The potential for metastasis appeared to be correlated with SATB1 expression levels, as SATB1-shRNA2 MDA express lower levels of SATB1 than SATB1-shRNA1 MDA cells (Fig.

2a). In further support of this observation, RT-PCR analysis of lungs revealed that the human SATB1 transcript levels were increased in two of the mice (#55-1 and #55-6) injected with SATB1-shRNA1 MDA cells (Fig. 4b). These two mice showed 22 and 50 metastatic nodules/lung while all other mice injected with SATB1 knockdown cells showed less than 5 nodules/lung. Thus, our data from experimental metastasis analyses indicate that SATB1 is necessary for the aggressive, highly metastatic phenotype of MDA-MB-231 cells and suggest that the levels of SATB1 also play an important role in the metastatic activity of cancer cells.

SATB1 overexpression in Hs578T cells increases metastasis

We next tested whether SATB1 overexpression could promote metastasis in another breast cancer cell line, Hs578T, in which SATB1 is expressed at a lower level than in MDA-MB-231 cells. When we forced express SATB1 at higher levels in Hs578T cells, their invasive activity *in vitro* was greatly increased (Supplemental Fig. 1e, f). When control Hs578T cells (2×10^6 cells/mouse) transfected with vector alone were injected into 6 mice (HS), only two mice developed metastatic nodules in the lung and in both cases there was only one nodule per mouse, consistent with the less aggressive nature of the Hs578T cells than the MDA-MB-231 cells (Fig. 4c, d). In contrast, Hs578T cells transfected with pLXSN-SATB1 and over-expressing SATB1 formed a greatly increased number of lung metastases in all mice (HS25), ranging from 25 to 157 metastatic nodules per lung. Of 6 mice injected with the SATB1-overexpressing cells, 3 mice developed over 120 metastatic nodules per lung. This number was equivalent to the number of metastases that formed in the lungs of mice injected with control MDA-MB-231 cells. Therefore, our results strongly suggest that SATB1 is not only required for, but induces breast cancer cell metastasis to lung (Fig. 4b, d).

Global changes in gene expression associated with SATB1 levels in cancer cells

Given that SATB1 can orchestrate the expression of a large number of genes in thymocytes, we investigated how loss of SATB1 might affect the expression profile of breast cancer cells. Microarray experiments revealed that after RNAi-mediated removal of SATB1 from MDA-MB-231 cells (the SATB1-shRNA1 and SATB1-shRNA2 MDA cell lines), the expression levels of ~1200 genes were either down- or up-regulated at least two-fold. We also compared the expression patterns of these genes in cells grown in either 2D or 3D cultures.

Genes whose expression levels were changed by loss of SATB1 were categorized based on the gene ontology (GO) annotation (Supplemental Fig. 2 and Supplemental Table 2). Many of these affected genes were found associated with cell cycle, transcription, cell-cell adhesion, tight junction, cell motility, cytokine activity/growth, and signal transduction such as Wnt signaling and TGF- β signaling pathways. Furthermore, among 155 genes which matched with the 231 genes that were previously identified from microarray analyses to be significantly associated with poor prognosis of breast cancer⁶, 55 of them (36%) were found to be altered in an SATB1-dependent manner from our studies with MDA-MB-231 cells on 3D cultures, and those genes up-regulated in aggressive cancer cells were also up-regulated by SATB1 and vice versa (Supplemental Fig. 3). The SATB1-dependent genes were also enriched for those genes known to promote either lung or bone metastasis in MDA-MB-231 cells as well (Supplemental Fig. 3).

Based on both the data from our microarray analyses and from the published literature on genes associated with breast cancer progression, we selected over 40 genes to confirm their SATB1-dependent expression by RT-PCR (Fig. 5 and Supplemental Fig. 4). In MDA-MB-231 cells, we found that SATB1 preferentially functions as a transcriptional enhancer, rather than as a repressor. Expression of many genes that are known to have important functions in promoting metastasis was found to be down-regulated upon SATB1 depletion. The SATB1-dependent genes associated with breast cancer progression include *SI00A4* (encodes Mts1 or metastasin) which has roles in metastasis²¹⁻²⁴ and angiogenesis^{25,26}; *matrix metalloproteases* (MMPs) 2, 3, and 9, which degrade extracellular matrix (ECM) and promote tumor invasion²⁷⁻²⁹; *tumor growth factor β 1* (TGF- β 1), which stimulates invasion³⁰; *connective tissue growth factor* (CTGF), which mediates angiogenesis³¹ and bone metastasis³²; and the tumor suppressor *BRMS1*³³ (Fig. 5a). When SATB1 is expressed, the tumor suppressor *BRMS1* is repressed, whereas all the other metastasis promoting genes in this group are upregulated. Significantly, SATB1 expression also correlated with upregulation of genes involved in epidermal growth factor (EGF) signaling³⁴, such as EGF receptor subfamily members *ERRB1*, *ERBB2* (also known as *HER-2* or *NEU*), *ERBB3*, *ERBB4*, the ligands *NRG* and *AREG*, and the *ABL1* oncogene, which has a role in EGF-induced-ERK signaling. *ERBB2*, the most oncogenic family member of ERBB protein, is an important regulator of breast cancer progression by coordinating the ERBB signaling network³⁴. Elevated expression of ERBB proteins are often

found in human cancer and drugs that intercept signaling generated from ERBB2 are in routine clinical application³⁵. Many tumor cells exhibit increased invasiveness in response to TGF- β 1 and increased levels of TGF- β 1 has been reported in most tumor types³⁰. These results show that SATB1 promotes the expression of a set of genes that are known facilitators of metastasis while downregulating tumor suppressor genes. The dramatic shift in the gene expression pattern in cancer cells that express SATB1 can cause these cancer cells to acquire an invasive and aggressive phenotype.

Consistent with reversion of cell morphology for SATB1-depleted MDA-MB-231 cells, genes whose expression is up-regulated in invasive breast cancer and products contribute in cell structure are all down-regulated by SATB1 depletion (Fig. 5a). These genes include an ECM protein, fibronectin (FN); an intermediate filament protein, vimentin (VIM)³⁶; cell-ECM interacting protein, β 4 integrin (ITGB4)³⁷. A nuclear structural protein, lamin A/C (LMNA), was similarly down-regulated by SATB1 depletion. Dysregulated expression in cadherin and catenins, which mediate cell-cell adhesion, has also been detected in breast cancer^{38,39}. OB-cadherin (CDH11), VE-cadherin (CDH5), and N-cadherin (CDH2) that are often up-regulated in invasive breast cancer were all repressed in SATB1 knock-down cells. Although SATB1 up-regulates the above described genes, for certain genes SATB1 acts as a repressor. These genes that are found de-repressed in SATB1 knock-down cells include CLDN1, a tight junction protein, which is known to be either lost or scattered in invasive tumors⁴⁰; β -catenin, a component of the cadherin-catenin complex and a critical member of the canonical Wnt pathway⁴¹; E-cadherin, an adherens junction protein and tumor suppressor^{38,42}. Loss of E-cadherin is a hallmark for epithelial to mesenchymal transition (EMT)—a process whereby epithelial cells layers lose polarity and cell-cell contacts and undergo a dramatic remodeling of the cytoskeleton, which is believed to contribute to the dissemination of carcinoma cells from epithelial tumors⁴³. SATB1 depletion from MDA-MB-231 cells resulting in upregulation of E-cadherin and restoration of acinar-like morphology strongly suggest that the EMT process was reversed.

We also examined whether the expression of the above group of genes was altered in Hs578T cells that overexpressed SATB1, compared to that in the parental Hs578T cell line (Fig. 5a). In addition to *BRMS1*, other tumor suppressors, *NM23* and *KISS1* were downregulated upon SATB1 overexpression. These three tumor suppressor genes reduce the metastatic

propensity of a cancer cell line *in vivo* without affecting its tumorigenicity⁴⁴. We also found that many of the same metastasis-related genes, including those of the EGF receptor, MMPs, and cell structure/adhesion families, were upregulated in SATB1-overexpressing cells. This is in contrast to the SATB1-shRNA1 and SATB1-shRNA2 MDA cells in which removal of SATB1 resulted in downregulation of these gene families. Genes confirmed to be SATB1-independent are shown in Supplemental Fig. 4a and genes whose expression is SATB1-dependent in either MDA-MB-231 cells or Hs578T cell systems are also shown in Supplemental Fig. 4b. β 4 integrin and laminin were both upregulated upon SATB1 overexpression which is consistent with increase in the aggressive phenotype for this cell line⁴⁵. Expression of *β -catenin*, *E-cadherin*, *claudin 1* and *BRM1*, was repressed in Hs578T cells that overexpressed SATB1, verifying that SATB1 acts as a repressor for these genes. We confirmed that *ERRB2* and *β -catenin* protein expression levels were correlated with their transcript levels by Western analysis of both MDA-MB-231 and Hs578T cells (Fig. 5b). SATB1 is therefore a global regulator of a large number of metastasis-related genes.

SATB1 directly regulates *ERRB2* and other cancer-related genes

To identify genes directly regulated by SATB1, we determined the *in vivo* binding status of SATB1 within genomic loci of 6 genes whose expression was correlated with SATB1 levels in cells. These genes are *ERBB2*, *Metastasin*, *ABL1*, *TGF- β 1*, *LaminA/C*, and *MMP3*, representing candidate genes directly regulated by SATB1 (Fig. 5a). Five genes whose expression was observed to be independent of SATB1 levels were selected as non-SATB1 target controls (*GAPDH*, *NRP1*, *TIMP1*, *ITGA5*, and *ITBG5*) (Supplemental Fig. 4a). For each of these selected genes, we analyzed a ~15 kb region upstream and downstream of the gene's first exon for SATB1 binding *in vivo*, looking for all potential SATB1 target sequences (BURs), promoter sequences (if known), regions containing CpG islands, and other control sequences that would not be predicted to bind SATB1 based on DNA sequence. Potential BURs could be identified by the genomic sequences characterized by the ATC sequence context. SATB1 binding to each of these candidate sites was confirmed by electrophoresis mobility shift assay (EMSA). To assess SATB1 binding to these loci *in vivo*, we employed the urea-ChIP method¹², in which chromatin was crosslinked, purified from the cell lines by urea-gradient

centrifugation, Sau3A digested, and SATB1-containing chromatin fragments were immunoprecipitated using an anti-SATB1 antibody.

By comparing the overall *in vivo* SATB1-binding statuses for the candidate SATB1-target gene loci versus the non-target control gene loci, we found a striking contrast in SATB1 binding patterns between the two groups (Fig. 6 a, b). In all six candidate target gene loci, virtually all SATB1-binding sequences, predicted based on the ATC sequence context and confirmed by EMSA, were bound to SATB1 *in vivo* (Fig. 6a). A similar binding pattern was detected for the *β-catenin* locus, whose expression was downregulated when SATB1 was overexpressed (data not shown). On the other hand, in non-target control gene loci, even though all of them contained many sequences intrinsically capable of binding to SATB1 *in vitro*, SATB1 was rarely found to be associated with them *in vivo* (Fig. 6b). The contrast in the *in vivo* binding frequencies of SATB1 between the two sets of genes indicates that there are at least two clearly different gene subgroups, that can be distinguished by the SATB1-binding status, regardless of whether SATB1 represses or activates expression of the genes. These results, taken together with SATB1-dependent expression, strongly suggest that SATB1 directly regulates expression of *ERBB2*, *Metastasin*, *ABL1*, *TGF-β1*, *LaminaA/C*, *MMP3* and *β-catenin*.

For genes directly regulated by SATB1, SATB1 binding does not occur exclusively at the sequences that have the capacity to bind SATB1. Some sequences near promoters or CpG islands that totally lack SATB1 binding potential based on EMSA can be bound *in vivo*. Such binding sites are found near promoters of *ABL1* (sites 1 and 6), *TGF-β1* (site 10), *Lamin A/C* (site 4) (Fig. 6a). The remaining SATB1 target genes tested here already have SATB1-binding sequences near promoters, and SATB1 binds to these sites *in vivo*. SATB1 binding *in vivo* to promoters or nearby sequences is another hallmark for direct target genes. This is because SATB1 binding indirectly to promoter/regulatory sequences has been found within other known direct target genes of SATB1, such as *Il2Ra*⁹ and *Il4*, *Il5*, *Il13*¹⁴. Such indirect binding by SATB1 presumably reflects the SATB1-mediated formation of a large genomic DNA/protein complex placing multiple genomic sites into close spatial proximity¹⁴. Many other metastasis or cancer-associated genes whose expression is SATB1 dependent are likely to exhibit similar pattern for *in vivo* association with SATB1.

DISCUSSION

Our results show that SATB1, once it is expressed and upregulated in breast cancer cells, acts as a genome organizer to regulate a specific set of genes important for cancer progression and metastasis, such as *ERBB2*, *TGF- β 1*, *metastatin*, and *MMPs*. Through overexpression and knock-down of SATB1 in different breast cancer cell lines, we have shown that SATB1 is both necessary and sufficient to induce aggressive phenotypes *in vitro* as well as metastasis to the lungs in mice. SATB1 is upregulated in aggressive breast cancer cell lines and aggressive primary tumor samples. Our data strongly suggest that SATB1 expression in primary tumors is an important determinant of their metastatic ability.

In order for breast cancer cells to acquire their invasive activity, they must upregulate genes important for cancer invasion, such as those that promote aggressive cell morphology, ECM-degrading enzymes, growth factors and receptors that accelerate cell proliferation, and cell-matrix adhesion factors that promote cell migration. They must also downregulate tumor suppressors. There are distinct gene expression profiles associated with different stages of cancer progression⁴⁶⁻⁴⁸, and some of the profiles can be used to predict a patient's prognosis⁴⁻⁶. Since studies have shown that such profiles can be detected in some primary tumors, even before the cells metastasize, there might be a 'global switch' that turns these genes on early in tumorigenesis, rather than accumulation of random mutations and gene dysregulation throughout cancer progression. Although it is unknown what mechanism initially triggers SATB1 expression, our results show that SATB1, when expressed in breast cancer, orchestrates expression of genes necessary for the acquisition and maintenance of aggressive phenotypes.

When SATB1 expression was knocked-down by RNAi in the aggressive breast cancer cell line MD-MB-231, we observed by DNA microarray that the expression pattern of ~1200 genes was altered compared to the parent cell line. The SATB1-dependent regulation of many genes in this profile occurs whether cells are grown in 2D or 3D cultures (Fig. 5). We have shown that SATB1 directly regulates *ERBB2*, *TGF- β 1*, *metastatin*, *lamin A/C*, *MMP-3*, *ABL-1* and *β -catenin*. We expect many more genes whose expression is SATB1-dependent are directly regulated by SATB1 as well. This is because all six genes we chose to analyze based on their SATB1-dependent expression were tethered to SATB1 at many positions in their gene loci, while all five genes whose expression was independent of SATB1 were not associated with SATB1 even though there were many potential SATB1-binding sites. It is particularly significant that *ERBB2* and *TGF- β 1* are SATB1 target genes because the ERBB and TGF- β

networks are implicated in multiple human cancers, and dysregulation of the many signaling pathways induced through either ERBB2 receptor tyrosine kinases or TGF- β can promote multiple properties of neoplastic cells, including excessive growth, invasion, metastasis, and angiogenesis^{30,34,49}. Many genes are expected to be controlled by such signaling network. For instance, it is known that the TGF- β 1/SMAD pathway activates CTGF. Thus, when TGF- β 1 expression is augmented by SATB1, it would promote CTGF expression³². Therefore, SATB1 can induce multiple gene activation cascades by directly regulating multiple key effectors for metastasis.

SATB1 has previously been shown to be a global regulator of gene expression, binding to numerous loci in thymocyte DNA, where it acts as in region-specific epigenetic modification of histones to mediate chromatin remodeling^{9,10}. However, it was not known whether SATB1 selectively binds to SATB1-binding sequences (ATC sequences) only within genes it directly regulates, or whether SATB1 can bind to any ATC sequence throughout the genome but recruits transcription factors only to a subset of them. This study showed that SATB1 binds specifically to the loci of the genes it controls, tethering multiple ATC sequences within each target gene loci to the SATB1 network. Our finding simultaneously suggests that there must exist a mechanism for protecting intrinsic SATB1-binding sequences in the non-target loci from binding to SATB1.

The feature that SATB1 binding occurs at multiple sites within each gene locus has an important biological significance in transcriptional regulation. Upon T cell activation, SATB1 has been shown to tether multiple positions in the cytokine gene cluster locus to the SATB1 cage structure to form a dense-loop configuration. Such chromatin structure places many ATC sequences and regulatory sequences in close proximity, creating a transcriptionally active chromatin structure at the cytokine gene cluster locus¹⁴. Based on analysis of SATB1 binding sites in breast cancer cell genomic DNA, it is likely that a similar dense-looping may also occur in some of the SATB1-directly regulated gene loci.

Interestingly, the group of genes regulated by SATB1 in breast cancer cells differs from that in thymocytes. For instance, T cell related genes, such as *Il2-Ra*, *Il-7R*, are ectopically expressed at high levels in SATB1-null thymocytes and some of them are directly regulated by SATB1 in thymocytes⁹. These T cell genes were expressed at very low levels in both control and SATB1-shRNA MDA cells. In contrast, breast cancer-related genes such as *ERBB2*, which

are upregulated by SATB1 in breast cancer cells, are expressed at very low levels in both wild-type and SATB1-null thymocytes (data not shown). Therefore, SATB1 appears to regulate distinct groups of genes in different cell types. Regardless of the specific cell type that expresses SATB1, the SATB1 network plays an important role in reprogramming chromatin organization and thus controlling expression of genes required for cells to gain new functions and/or to change their fate, such as differentiation, activation, or acquisition of metastatic activities. Further studies are required to determine the mechanisms by which SATB1 controls expression of different sets of genes in different cell types as well as an upstream regulator(s) of SATB1.

We present a new model of gene regulation during tumor progression, in which the nuclear architecture regulator SATB1 can control the expression of large numbers of genes to change the phenotype of a cancer cell. This finding supports emerging links between chromatin structure modifying/remodeling enzymes and cancer^{50,51}. Our data also show that in breast cancer cells SATB1 is bound to the loci and thereby regulates the expression of genes known to be important for breast cancer progression, including *ERRB2*, *TGF- β 1* and metastasin. Tumor cells are thought to accumulate genetic and epigenetic alterations that dynamically affect their transcriptional profiles. SATB1 might be a major switch that, when expressed or upregulated, cancer cells will be re-programmed at the level of chromatin organization, epigenetic modifications, and gene expression to gain metastatic capability. The discovery of the role of SATB1 in metastasis of breast cancer cell lines, if applicable as expected to metastatic cells *in vivo*, will change the paradigm of how we view metastasis in breast and possibly other organs. As such, SATB1 would be a useful marker of metastatic potential of breast tumors, as well as an important therapeutic target.

Materials and methods

Cells and Cell culture

Breast cancer cell lines, MCF-7, MDA-MB-453, SkBR3, Hs578T, BT549, MDA-MB-435, and MDA-MB-231 were obtained from American Type Culture Collection (Rockville, MD). Breast cancer cell lines MCF10A, SUM225, HCC202, BT474 were provided by Joseph W Gray and Richard M Neve (Lawrence Berkeley National Laboratory; Berkeley, CA). The cell pellets from immortalized mammary epithelial cell lines 184A1, 184AA2, 184V were obtained from M

Stamper (Lawrence Berkeley National Laboratory; Berkeley, CA). The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Life Technologies, Inc.; Grand Island, NY) at 37°C in 6% CO₂. MCF10A cells were maintained in DMEM/Ham's F12 medium supplemented with 5% horse serum (Invitrogen), 20ng/ml of EGF, 0.5µg/ml of hydrocortisone, 100ng/ml of cholera toxin, and 10µg/ml insulin (all from Sigma, St Louis, MO). Cell cultures were carefully monitored to avoid cell-to-cell contact/growth inhibition and potential transformation. Cell cultures were passed at 4-5 day intervals.

Human primary breast carcinomas, benign breast lesions, and normal breast tissues surgically removed and snap frozen in liquid nitrogen were obtained from the Cooperative Human Tissue Network (Nashville, TN). All of these tissue samples were processed for pathological analysis according to standard diagnostic criteria ⁵².

SATB1 knockdown and overexpressing cell preparation

Two shRNAs were designed according to SATB1 sequence (NM_002971) using siRNA Target Finder (Ambion, Austin, TX); The sequences of each oligoduplex were targeted as follows: shRNA₂₄₂₃, 5'-GGATTTGGAAGAGAGTGTC-3', or shRNA₂₅₉₅, 5'-GTCCACCTTGCTTCTC TC-3'. The oligoduplexes were cloned into the plasmid pSUPER (Oligoengine, Seattle, WA). Prepared DNAs were transfected into the aggressive breast cancer cell line MDA-MB-231 by lipofectamine 2000 (Invitrogen) and successfully transfected cells were selected by puromycin at 2 µg/ml or G418 at 1.5 mg/ml from 24 h after transfection. Single MDA-MB-231 cell clone stably expressing either shRNA₂₄₂₃ or shRNA₂₅₉₅ is designated SATB1-shRNA1 MDA or SATB1-shRNA2 MDA cells, respectively. For overexpression of SATB1, full length of SATB1 including 3'UTR was cloned into retroviral vector pLXSN (Clontech, Mountain View, CA), and the viral solution was produced using PT67 package cell lines. Hs578T cells were infected with this viral solution, and stably infected cells were selected by G418 at 0.8mg/ml for 5 days. The status of SATB1 level in manipulated MDA-MB-231 and Hs578T cells were examined by Western blot and real-time RT-PCR.

Cell growth assay

Total 2×10^4 cells were plated on plastic dishes, and cultured for up to 10 days at 37 °C in 6-well plates (2D culture). Growth medium were renewed every 4 days. Cells were harvested by trypsin treatment, and counted at each time point using a cell counter (Beckman Coulter, Inc.; Fullerton, CA) and haematocytometer. Total 5×10^3 cells were seeded on Matrigel (BD Biosciences, Inc.; Bedford, MA) coated 24-well plates (3D culture) as triplicate and incubated for up to 10 days at 37°C. Cells were treated with dispase (BD Biosciences, Inc.) for 2h at 37°C to be isolated from Matrigel, incubated with trypsin for further 5 min, and counted using a haematocytometer¹⁷. Samples were analyzed in triplicate at 0, 2, 4, 6, 8, and 10 days after cell culture was initiated. Trypan blue exclusion analysis indicated that 99–100% of the cells were viable.

Chemoinvasion assay

Boyden chamber chemo-migration assays⁵³ were performed using a 24-well chemotaxis chamber (BD biosciences, Inc.). Breast cancer cells were seeded in triplicate at 50,000 cells/well onto the upper chambers with a 8µm polycarbonate filter membrane coated with diluted Matrigel (10-25%) (BD biosciences, Inc.), and incubated at 37°C in humidified 5% CO₂ for 20 hrs. Conditioned media derived from NIH3T3 fibroblast cultures was used as a chemoattractant in the lower chambers. The migrated cells on underside of chambers were fixed in 10% (wt/vol) buffered-formalin and stained with crystal violet. After removal of cells remaining in the top chamber with a cotton swab, the numbers of cells that had migrated through the pores were assessed by light microscopy.

Matrigel outgrowth assay

Total 1×10^4 cells mixed with Matrigel (5 mg/mL) were plated in 6-well plates pre-coated in a thick layer (1mm) of Matrigel. Solidified Matrigel was overlaid with Growth medium (DMEM), and renewed at every 4 days. Colony formation and cell morphology was observed under light microscopy after 3 days to 14 days.

Soft agar assay

2.5 mL of 0.5% agarose in DMEM/F12 was plated on 60mm dishes and then overlaid with 1×10^4 cells in DMEM/F12 that contained 5% fetal bovine serum and 0.3% agarose. Cultures were maintained for 20 days. Colonies that exceeded the minimum diameter of 50 μm were scored as positive. Experiments were done in triplicate and repeated three times. One representative experiment is shown.

Western blot analysis

Proteins extracts were prepared from breast cancer cell lines or mammary tumor tissues by homogenization in lysis buffer (20mM HEPES (pH7.9), 25% glycerol, 0.5N NaCl, 1mM EDTA, 1% NP-40, 0.5mM DTT, 0.1% deoxycholate) containing the protease inhibitors (Roche Molecular Biochemicals USA, Indianapolis, IN). The homogenates were centrifuged at 100,000rpm and the pellets were discarded. Protein concentrations in the supernatants (lysates) were determined by BCA protein assay kit (Bio-Rad, Hercules, CA). 40 μg of cell lysates were subjected to electrophoresis through 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore, Billerica, MA). The membrane was blocked by incubation with 5% bovine serum albumin (BSA, fraction V, Sigma) in TST (20mM Tris-HCl, pH7.4, 0.5M NaCl, and 0.05% tween 20). The blots were then incubated with anti-SATB1 (1:500, BD Bioscience), anti- β -catenin (clone 14, 1:500, BD Bioscience), anti-ERBB2 (1:200, Lab Vision Corp., Fremont, CA), anti- α -tubulin (1:2000, Sigma) and anti-GAPDH (1:2000, Chemicon International, Inc.; Temecula, CA) antibodies for overnight at 4°C chamber. Then, the blots were incubated with appropriate peroxidase-conjugated secondary antibodies (1:10,000 diluted in TST) and detected by using SuperSignal chemiluminescence kit (Pierce, Rockford, IL).

Microarray

The concentration and purity of total RNA was measured by spectrophotometry at OD 260/280 and the quality of the total RNA sample was assessed using an Agilent Bioanalyzer with the RNA6000 Nano Lab Chip (Agilent Technologies, Palo Alto, CA). Biotin-labeled cRNA was prepared by linear amplification of the Poly(A)⁺ RNA population within the total RNA sample. Briefly, 2 μg of total RNA was reverse transcribed after priming with a DNA oligonucleotide containing the T7 RNA polymerase promoter 5' to a d(T)₂₄ sequence. After second-strand cDNA synthesis and purification of double-stranded cDNA, in vitro transcription was performed

using T7 RNA polymerase in the presence of biotinylated UTP. 10 µg of purified cRNA was fragmented to uniform size and applied to CodeLink Human Whole Genome Bioarrays (GE Healthcare, Little Chalfont, UK) in hybridization buffer. Arrays were hybridized at 37° C for 18 hrs in a shaking incubator. Arrays were washed in 0.75 x TNT at 46° C for 1 hr and stained with Cy5-Streptavidin dye conjugate for 30 min. Dried arrays were scanned with a GenePix™ 4000B scanner. Arrays were processed with CodeLink Expression Analysis software (GE Healthcare) and data was analyzed with GeneSpring software (Silicon Genetics). To compare individual expression values across arrays, raw intensity data from each gene was normalized to the median intensity of the array. Only genes that have values greater than background intensity in at least one condition were used for further analysis. For functional profiling on the basis of gene ontology terms with obtained data, we used the web-based software tool Onto-Express and Pathway-Express (<http://vortex.cs.wayne.edu/>)⁵⁴.

Immunofluorescence staining

Cells were plated on chamber-slides (Lab-Tek), fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X100, and blocked in 5% BSA. Staining for SATB1 and focal adhesion complexes was performed by incubating with anti-SATB1, anti-β-catenine (clone 14), anti-integrin α6 (CD49f, all from BD Biosciences) antibodies, and fluorescein phalloidin for F-actin staining (Invitrogen Molecular Probe) for overnight at 4°C. For immunohistochemical analysis, we obtained breast carcinoma Tissue Microarray from US BioMax (Rockville, MD). After deparaffinization, slide was boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 20 min. Tissue sections were incubated in 2% BSA/5% normal goat serum/0.1% Triton X-100 for 1 hour, and then subsequently reacted with primary antibodies against SATB1 (BD Biosciences) in blocking buffer for overnight at 4°C. Staining was detected with secondary Alexa Fluor 488 and/or Alexa Fluor 594 Abs (Molecular Probes). Cells were mounted in fluorescent mounting medium containing DAPI (Vector Laboratories).

We collected images by a DeltaVision microscope according to the manufacturer's instruction and processed with SoftWoRx software (Applied Precision, Issaquah, WA).

Lung metastasis analysis

MDA-MB-231 breast cancer cells, control cells, SATB1-shRNA1 MDA and SATB1-shRNA2-MDA cells (1×10^6 cells in 0.2ml PBS) and Hs578T control cells and SATB1 overexpressed Hs578T cells (2×10^6 cells in 0.2ml PBS) were injected intravenously into the lateral tail vein of 6 week-old athymic mice (NCR/nu-nu, Taconic, Hudson, NY) to evaluate lung colonization because metastases from an orthotopically growing tumor are relatively rare from these cell lines. Each treatment group consisted of 5-6 mice. At termination, the lungs were removed and fixed with 10% (wt/vol) neutral-buffered formalin. The number of lungs with surface metastases were determined, as well as the number of surface metastases per lung by examination under a dissecting microscope, as described elsewhere⁵⁵

RT-PCR and real-time RT-PCR

To evaluate the change of gene expression level depending on SATB1 status, semi-quantitative or real-time RT-PCR analysis of selected genes was performed (primer sequences are available upon request). Total RNA was extracted for cell lines using TRI reagent (Sigma) followed by RNA clean-up with RNeasy Mini kit (Qiagen, Valencia, CA). For semi-quantitative RT-PCR, 5 μ g of total RNA was reverse-transcribed into single stranded cDNA using Superscript II RNaseH- reverse transcriptase (Invitrogen) according to the protocol supplied with the kit. We carried out PCR reactions as described in previous paper¹⁰. PCR cycle was controlled starting from 25 to 40 cycles upon gene-specific primers (20ng of cDNA/reaction). Each cycle consisted of the following steps, using GeneAmp PCR system 9700 (PerkinElmer Inc., Fremont, CA); 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. PCR products were separated on 1.5% agarose gels and visualized them by staining with ethidium bromide. SYBR Green PCR Core Reagents system was used for real-time monitoring of amplification on ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). We employed absolute quantification method to quantify target DNA fragments in triplicate with following cycling condition; 95°C for 2 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

Chromatin immunoprecipitation assay (ChIP) for in vivo DNA binding

Urea-ChIP experiments were performed as previously described with modification^{56,57}. Cross-linked chromatin by formaldehyde was isolated from MDA-MB-231 and SATB1-shRNA MDA cells (M5-5), and then purified further using urea-gradient ultracentrifugation. After 60U of

Sau3A1 digestion, we performed immunoprecipitation of 30 μg of cross-linked chromatin against anti-SATB1 antibody (BD Biosciences) and purified mouse IgG₁ (Sigma) as a control. We reversed the ChIP samples with 100 $\mu\text{g}/\text{ml}$ RNase A and 250 $\mu\text{g}/\text{ml}$ proteinase K treatment, followed by incubation at 65°C for 6 hrs. We carried out PCR reaction using reverse cross-linked chromatin with AmpliTag-Gold DNA Polymerase (Applied Biosystems) in following cycling condition; 95°C for 10 min and 35-40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 20 s, and extension at 72°C for 30 s using GeneAmp PCR system 9700 (PerkinElmer Inc.). We designed the primer sequences mainly focused on the promoter regions of each gene (covering ~15kb) using Vector NTI software (Invitrogen), and listed in Supplemental Table 4. We performed gel mobility shift assay (EMSA) to determine the *in vitro* binding ability between SATB1 and SATB1 binding sequences *in vivo*, which we detected in urea-ChIP assay.

Statistics

Fisher's exact test was performed using InStat 3 software v3.6 (GraphPad Inc., San Diego, CA) for data categorized into 2 X 2 contingency tables.

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Figure Legend

Figure 1. SATB1 expression is detected in highly aggressive cancer cell lines and primary tumor samples, but not in benign and normal samples.

a. Western blot analysis of SATB1 levels (upper panel) was performed with normal mammary epithelial cells, HMEC, immortalized mammary epithelial cells (184A1, 184AA2, 184V and MCF-10A), non-aggressive breast cancer cell lines (BT474, MCF7, MDA-MB-435, SUM225

and SKBR3) aggressive breast cancer cell lines (HCC202, Hs578T, MDA-MB-435, BT549, MDA-MB-231). SATB1 expression was detected only in aggressive breast cancer cell lines. Tubulin was used as quantity control. **b.** Western blot analysis of representative SATB1 in human primary breast tumor samples. Silver staining was used to control for loading of samples. The pathological summaries of each specimen are listed in Supplementary Table 1. Of 28 tumor samples, 15 that were highly metastatic and lymph node-positive tumors were strongly positive for SATB1 expression with a high statistical significance at $P < 0.0001$. **c.** Fluorescence immunostaining of normal and metastatic ductal carcinoma (MDC) mammary tissues from a tissue array (Biomax) using an antibody against SATB1 (red). DNA was counterstained with DAPI. Tissues structures were examined by hematoxylin and eosin (H& E). The TNM stages (T, the size or spread of primary tumor; N, presence or absence of tumor cells in axillary lymph-node; M, presence or absence of distant metastasis), providing information on anatomic extent of malignant disease are indicated⁵⁸.

Figure 2. RNAi-mediated depletion of SATB1 reduces the proliferative activity, invasiveness and anchorage-independent growth of aggressive MDA-MB-231 cells.

SATB1 was depleted from cells by RNAi, using short hairpin-interfering RNAs (shRNA) that targeted two sequences (one in the coding region and the other in the 3'UTR region) of *SATB1*. **a.** Western blot analysis and quantitative RT-PCR analysis show that SATB1 expression was greatly reduced in MDA-MB-231 cells stably transfected with the pSUPER-puro construct expressing the shRNA against either the coding region (SATB1-shRNA1) or the 3'UTR (SATB1-shRNA2) compared to that in parental MDA-MB-231 cells. SATB1 expression levels remained unchanged in MDA-MB-231 cells that stably expressed an shRNA whose sequence did not match any known human gene (control shRNA). **b.** The rate of proliferation of the parental MDA-MB-231 cells, control cells (expressing control shRNA), SATB1-shRNA1 MDA cells and SATB1-shRNA2 MDA cells grown on either plastic (2D) or matrigel (3D) culture plates was determined. **c.** The invasiveness of the parental MDA-MB-231 cells, control cells, SATB1-shRNA1 and SATB1-shRNA2 MDA cells was measured by Matrigel-coated Boyden Chamber assay. Each data point represents the mean \pm S.D. of results obtained from three independent experiments. **d.** Anchorage-independent growth of control cells and SATB1-shRNA1 MDA cells was examined by culturing these cells in soft agar (20 days) and assessing colony formation.

Representative photos of soft agar colonies are shown. The number of cell colonies presented is the mean value of colony counts from three dishes.

Figure 3. Reversion of cell morphology and restoration of polarity on 2D and 3D culture following SATB1 depletion from highly aggressive MDA-MB-231 cells.

a. Phase contrast micrographs of MDA-MB-231 control cells (control shRNA) or SATB1-shRNA1 MDA cells (SATB1-shRNA1) cultured on plastic (2D) and matrigel (3D). Cells grown in 2D were immunostained with anti- β catenin (green) and counterstained with DAPI. Cell junctions in SATB1-shRNA1 MDA cells grown in 2D culture appear mostly disrupted. We observed major differences in cell morphology when SATB1-shRNA1 MDA cells were grown in 3D culture, compared to control cells. Whereas control cells exhibited a spindle-like fibroblastic morphology, SATB1-shRNA1 MDA cells had a cobble-stone like morphology. **b.** Immunostaining with antibodies against F-actin, β -catenin, integrin α 6 (all green), and counterstained with DAPI (DNA, blue) indicated that SATB1-shRNA1 or SATB1-shRNA2 MDA cells grown in 3D culture had an organized and polarized morphology, forming acinus-like structures. In contrast, control cells in 3D lost polarity and showed disorganized morphology with altered lateral and basement membrane structures. **c.** Control cells grown on either 2D or 3D were stained with anti-SATB1 antibody (red) and counterstained with DAPI (blue). Cells grown on 2D typically showed a punctuated distribution, while 3D showed a network-like distribution of SATB1. Images were captured by a DeltaVision fluorescent microscope. Bars=15 μ m

Figure 4. SATB1 expression in breast cancer cells is necessary and sufficient for survival in circulation and colonization in lungs,

a. RNAi-mediated depletion of SATB1 inhibited the ability of MDA-MB-231 cells to metastasize to lungs of nude mice. 1×10^6 cells MDA-MB-231 cells expressing control-shRNA, SATB1-shRNA1 or SATB1-shRNA2 MDA cells were injected into the tail veins of each mouse, and lungs were examined for metastatic nodules (arrows) 9 weeks later. Representative photos are shown. **b.** Total numbers of metastatic lung nodules from individual mice were counted under a dissection microscope. For lungs of representative mice indicated, human SATB1 expression levels in human breast cancer cells colonized in lungs were analyzed by RT-PCR

using human GAPDH as a loading control, with the use of human SATB1 and GAPDH specific oligomers. The specificity of these oligomers for human genes is shown by the absence of RT-PCR signals for mouse thymocytes (Thy). **c.** Overexpression of SATB1 promotes the ability of Hs578T cells to metastasize to lungs. 2×10^6 Hs578T cells transfected with vector alone (control) or with an SATB1 expression construct (pLXSN-SATB1) were injected into each mouse, via the tail vein, and lungs were examined 9 weeks later. Representative photos from three independent mice are shown. **d.** Total number of metastatic lung nodules formed in lungs of mice injected with Hs578T cells that overexpress SATB1 (HS25) and controls (HS). Similar to b), human SATB1 expression was analyzed by RT-PCR for representative mice indicated.

Figure 5. SATB1 induces global changes in gene expression.

a. Semi-quantitative RT-PCR analyses were performed to assess the expression levels of the genes known to be involved in metastasis suppression or promotion. RNA was prepared from two independent cell clones (control 1 and 2) isolated from MDA-MB-231 cells expressing control shRNA, SATB1-shRNA1 MDA cells and SATB1-shRNA2 MDA cells grown in 2D and 3D cultures. RT-PCR analyses were also performed for two independent Hs578T cell clones expressing control vector (control 1 and 2) and two independent cell clones (SATB1-1 and SATB1-2) both carrying the SATB1 expression construct (pLXSN-SATB1) grown on 2D culture. PCR conditions were optimized to detect the SATB1-dependent up- or down-regulation of expression of each gene analyzed in either MDA-MB-231 cells or Hs578T cells. The expression levels of the control (GAPDH) remained unchanged under all PCR conditions. A brief function of each gene product is provided. SATB1 acts as a transcriptional repressor for *BRMS1*, *E-cadherin (CDH1)*, *β -catenin (CTNNB)*, *claudin 1 (CLDN1)* (bottom four rows), but it functions as a transcriptional enhancer for the remaining genes listed here. GAPDH expression levels remained constant in all cell types. Expression levels of all other genes examined are shown in Supplemental Fig. 4. **b.** Protein expression levels of ERBB2 and β -catenin were analyzed by Western blot in parental MDA-MB-231 cells, control MDA-MB-231 cells (control shRNA), SATB1-shRNA1, and SATB1-shRNA2 MDA cells. GAPDH levels were used as loading control.

Figure 6. SATB1-binding patterns of gene loci are different between SATB1-dependent and independent gene groups. Chromatin immunoprecipitation (ChIP) was performed with either anti-SATB1 antibody or IgG on crosslinked cells using urea-gradient purified Sau3A-digested chromatin fragments (urea-ChIP assay). Primer sets were designed to cover ~15 region (indicated by number), including sites near or within the promoter of each gene as well as all genomic sequences that may have intrinsic potential to bind SATB1 due to the presence of the ATC sequence context. PCR amplification results from SATB1 ChIP samples of either MDA-MB-231 cells (MDA231-SATB1) or SATB1-shRNA MDA cells (M5-5-SATB1) IgG ChIP samples of (MDA231-IgG or M5-5-IgG) and input DNA (MDA231-Input or M5-5-Input), using the primer sets indicated by number below each map are shown. Input DNA was purified from Sau3A-digested crosslinked chromatin without immunoprecipitation and used to control for amplification efficiency of primers. The identical series of experiments were conducted in parallel using an MDA-MB-231 cells and SATB1-shRNA1 MDA cells (M5-5). The SATB1 binding activity of each fragment was confirmed by an electrophoresis mobility shift assay (EMSA), using bacterially produced recombinant SATB1 protein (strong binding sites, red bars; weak binding sites, yellow bars; and no binding, black bars). The fragments that bind to SATB1 *in vivo* are indicated by red star and also by red bar under ChIP. **a.** A group of genes whose expression was changed by both shRNA-mediated removal of SATB1 in MDA-MB-231 cells and by SATB1-overexpression in Hs578T cells, compared to control cells **b.** A group of genes whose expression was independent of SATB1 expression levels in cells. Gene structure is based on the data from USCS (<http://genome.ucsc.edu/>). Green,, CpG island; Blue, promoter region; light blue, transcription factor binding sites; Pink, exons.

Supplementary Table 1. The summary of pathological information of human primary breast tumor specimens which were used in this study.

Supplementary Table 2. The list of genes whose expression is SATB1 dependent in 2D or 3D culture conditions categorized in terms of biological pathway.

Analysis of functional profile heterogeneity within the genes regulated by SATB1 was performed using web-based analysis tool, Pathway-express (<http://vortex.cs.wayne.edu>). Among 1200 genes either up or down-regulated by at least 2 fold in an SATB1-dependent

manner in MDA-MB-231 cells, we found a total of 354 up-regulated genes and 267 down regulated genes that were involved in representative biological pathways based on KEGG (Kyoto Encyclopedia of genes and genomes) database. Genes whose expression was altered depending on SATB1 are mainly involved in the pathways of cell cycle, adherens junction, cytokine-receptor interaction, intracellular signaling, apoptosis and MAP kinase signaling.

Supplementary Figure 1. RNAi-mediated SATB1 depletion of BT549 and SATB1 overexpressed Hs578T cells exhibit changes in the cell morphology on Matrigel, invasiveness and anchorage-independent growth. **a.** SATB1 was depleted from aggressive breast cancer cell line BT549 using shRNA that targeted the sequences in the coding region (SATB1-shRNA1) or 3'UTR region (SATB1-shRNA2) of SATB1. Western blot analysis show that SATB1 expression was greatly reduced in both of BT549 cells stably transfected with pSUPER-puro-shRNAs compared to that in parental cells. **b.** Phase contrast micrographs of BT549 cells expressing either control-shRNA or shRNA against SATB1 (SATB1-shRNA1 and SATB1-shRNA2) on Matrigel after 5 days of culture. We observed the major differences in cell morphology in SATB1 depleted BT549 cells compared to control cell. Whereas control cells showed a spindle-like morphology, which is typical for aggressive breast cancer cells, SATB1-depleted BT549 cells had a cobble-stone like morphology, which is typical for normal or non-aggressive cancer cells. **c.** Anchorage-independent growth of control cells and SATB1 shRNA1 BT549 cells was examined by culturing these cells in soft agar for 20 days and assessing colony formation. Representative photos of soft agar colonies are shown. The number of cell colonies presented is the mean value (\pm S.D.) of colony counts from three dishes. Depletion of SATB1 prevented colony formation of BT549 cells in soft agar. **d.** The invasiveness of the parental BT549 cells, control cells, shRNA1 and shRNA2 cells was measured by Boyden Chamber assay. The invasive capacity *in vitro* of SATB1 depleted BT549 cells was significantly reduced compared to control BT549 cells. **e.** A breast cancer cell line, Hs578T cells were transfected with pLXSN-SATB1 construct to over express SATB1. The Western blot analysis verified increase in SATB1 protein levels in stably transfected two single clones. **f.** The *in vitro* invasiveness of the parental Hs578T cells, control, and two independent clones overexpressing SATB1 (SB25 and SB18) was measured by Boyden Chamber assay. The invasive capacity of SATB1-overexpressing Hs578T cells increased by 3-fold compared to their parental and control cells.

Supplementary Figure 2. Functional profiles of the genes regulated by SATB1 in MDA-MB-231 cells. Functional profiles using Gene Ontology terms for biological process and molecular function were constructed for SATB1-dependent up- and down-regulated genes (>2 fold) in either 2D or 3D cultures by Onto-Express (OE, <http://vortex.cs.wayne.edu/ontoexpress>) using the initial pool of 20,000 genes (Codelink from Amersham) as the reference set. The profiles for a total of 648 upregulated genes (red bar) and 519 down-regulated genes (green bar) are shown. We showed the functional categories of biological process that were significantly represented by SATB1-dependent genes with the statistical significance of $p < 0.05$ or represented by more than five genes. The results show that SATB1-dependent genes are highly represented in most of biological processes postulated to be associated with cancer including the positive control of cell cycle, cell proliferation, cell adhesion, signal transduction, cell-cell signaling and transcriptional regulation. The representative genes were listed as red (up-regulated) and green (down-regulated).

Supplementary Figure 3. Comparison with the prognosis signature genes with SATB1-regulating genes in microarray. **a.** The microarray data sets from MDA-MB-231-control shRNA/ SATB1-shRNA were compared with the data from van't Veer L.J. *et al.* for prognostic signature genes⁶. Among 231 genes for poor prognostic signature markers, 155 genes matched with CodeLink human 20K array probe sets (Amersham). About 36% (55 genes out of 155 genes total) of these markers were regulated by SATB1. **b.** The genes regulated by SATB1 were compared with the lung-metastasis promoting signatures by MDA-MB-231 cells, which had been previously reported⁵⁹. 89 marker genes were matched with CodeLink human 20K array probes, and 29 representative genes (33%) are found to be affected by SATB1 status. **c.** 127 bone metastasis marker genes were compared with matched 94 CodeLink human 20K genes, and selected 25 genes were shown (27%). Expressional fold ratio was converted to color-scale as shown in bottom.

Supplementary Figure 4. RT-PCR analysis of SATB1-independent and dependent genes. Semi-quantitative RT-PCR analyses were performed to assess the expression levels of the genes

known to be involved in metastasis suppression or promotion. RNA was prepared from two independent cell clones (control 1 and 2) isolated from MDA-MB-231 control cells, SATB1-shRNA1 and SATB1-shRNA2 MDA cells grown in 2D and 3D cultures. RT-PCR analyses were also performed for two independent Hs578T cell clones expressing control vector (control 1 and 2) and two independent cell clones (SATB1-1 and SATB1-2) both carrying the SATB1 expression construct (pLXSN-SATB1) grown on 2D culture. PCR conditions were optimized to detect the SATB1 dependency for each gene individually in either MDA-MB-231 cells or Hs578T cells. The expression levels of the control (GAPDH) remained unchanged under all PCR conditions. A brief function of each gene product is provided. **a.** Semi-quantitative RT-PCR analyses showed that the expression level of genes shown here remained constant in all cell types. **b.** Expression levels of all other genes examined are shown. Among which Twist is known to induce intravasation of metastatic tumor cells without affecting their proliferation or survival⁶⁰. However, we find that Twist is expressed at very low levels in MDA-MB-231 cells and Hs578T cells and that Twist expression remained low in all cell clones which we tested.

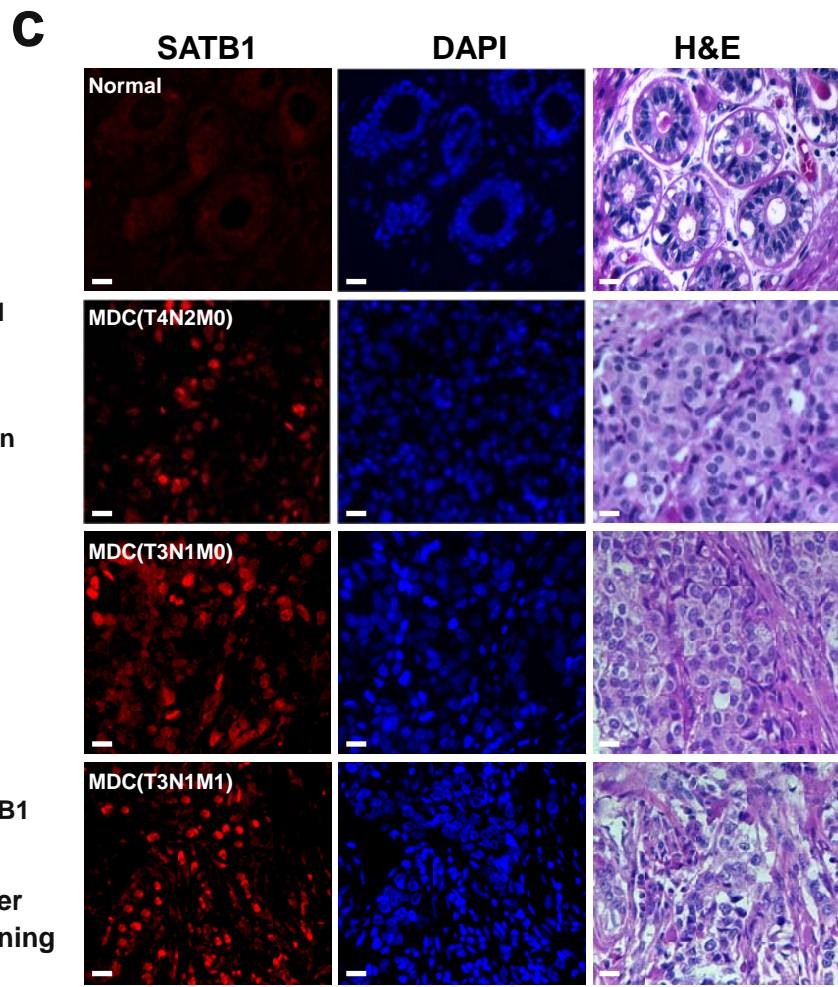
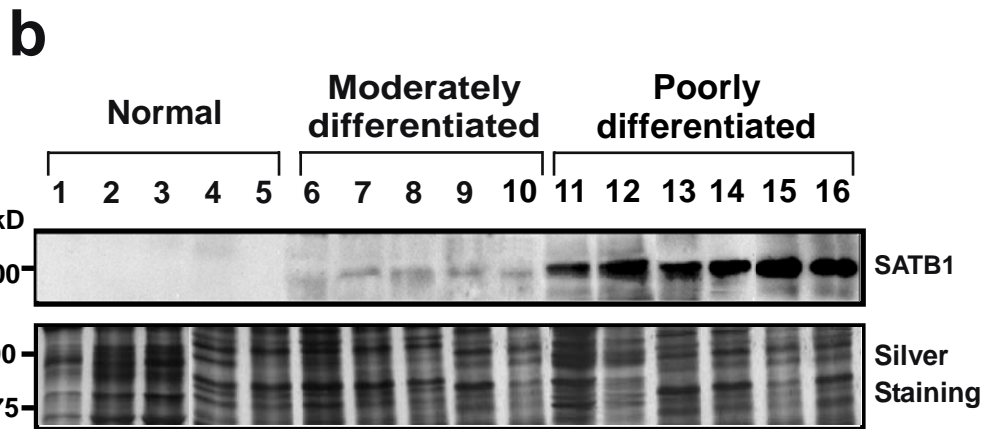
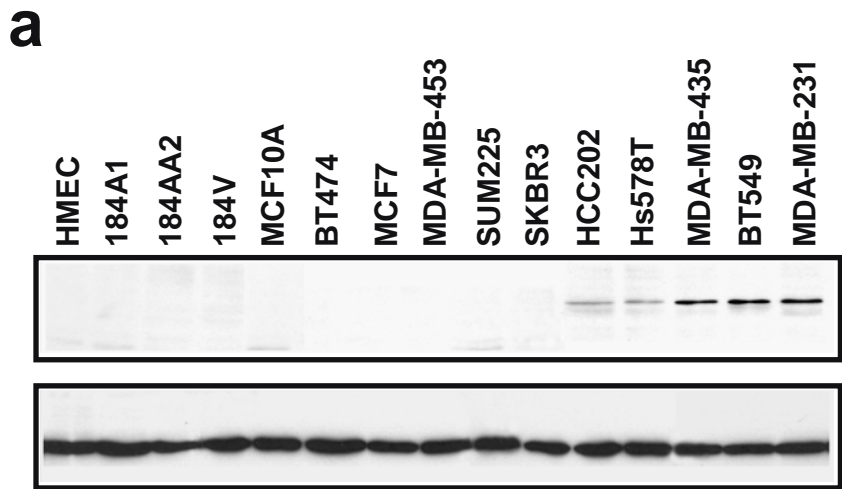
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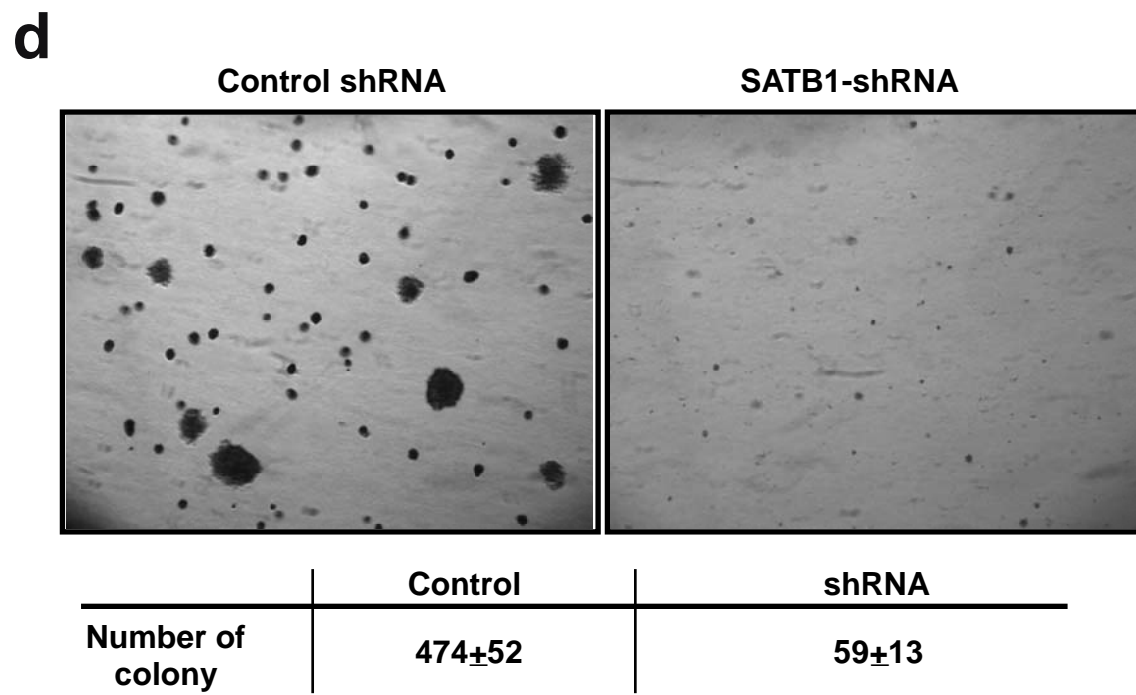
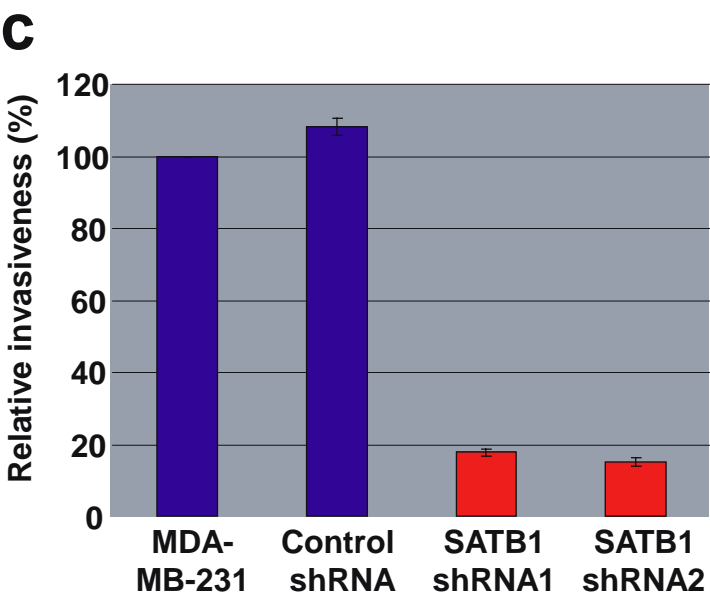
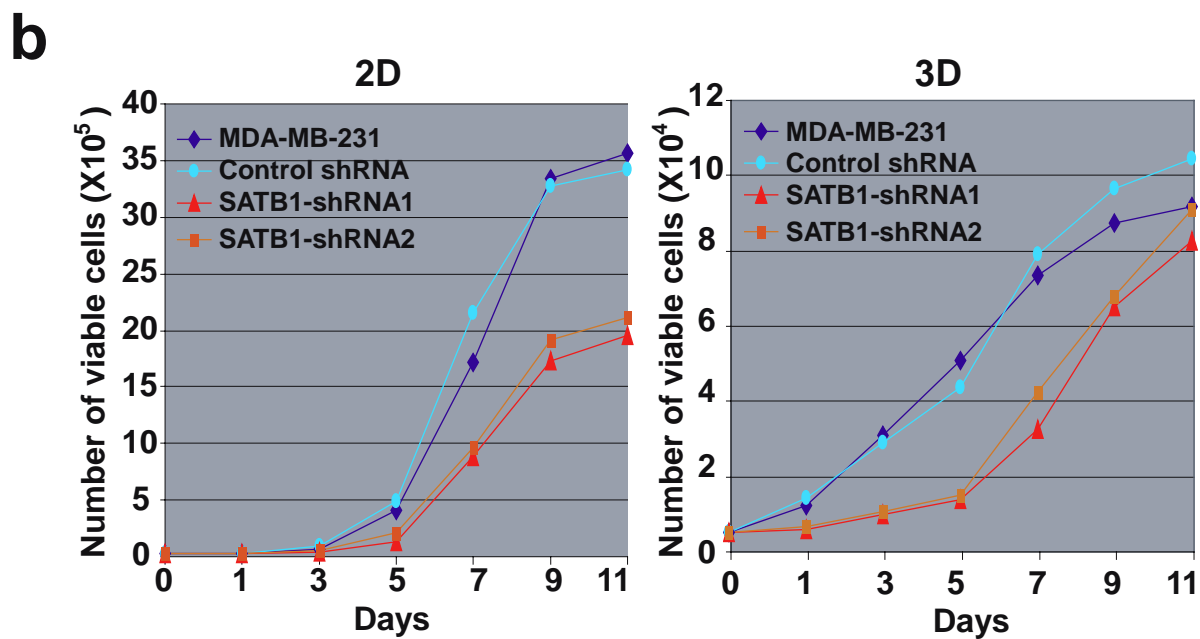
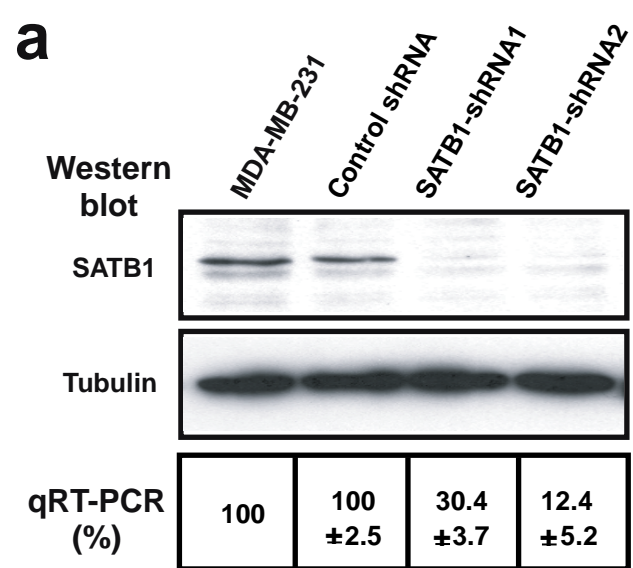
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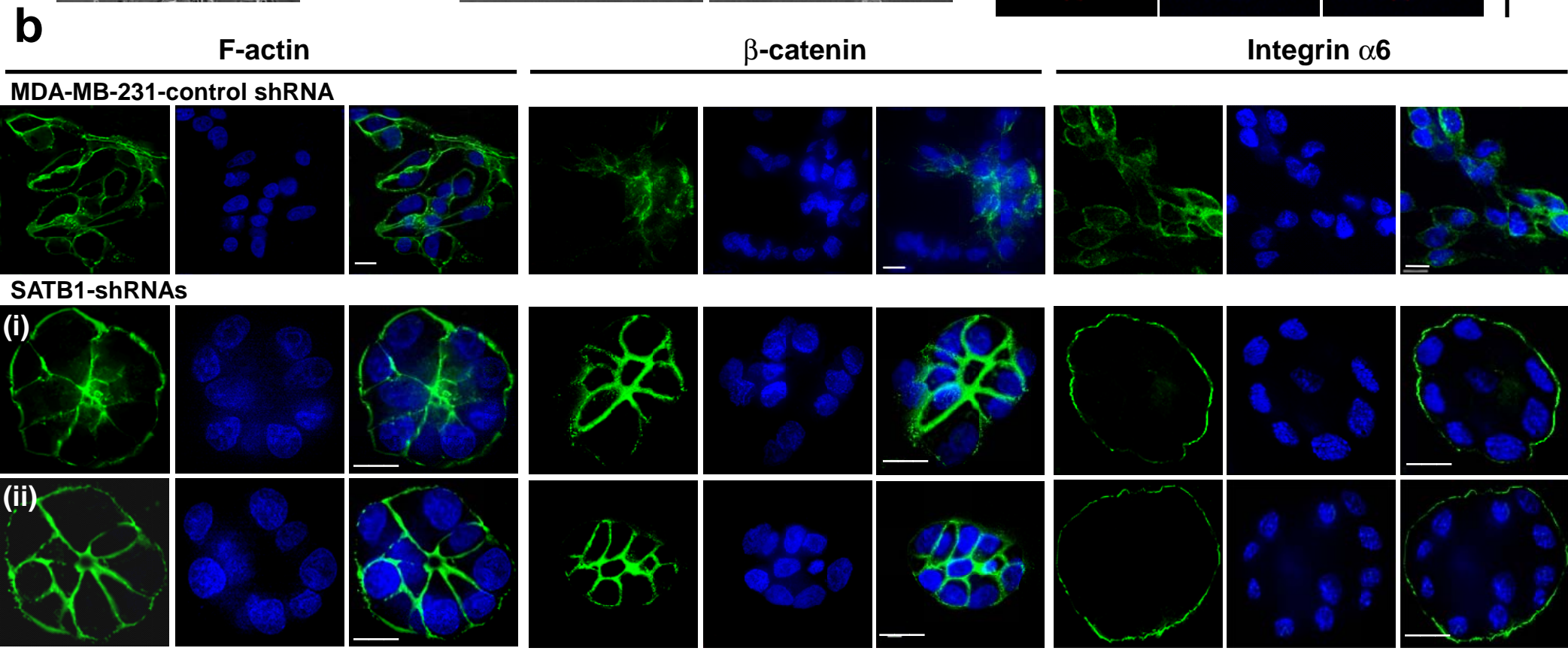
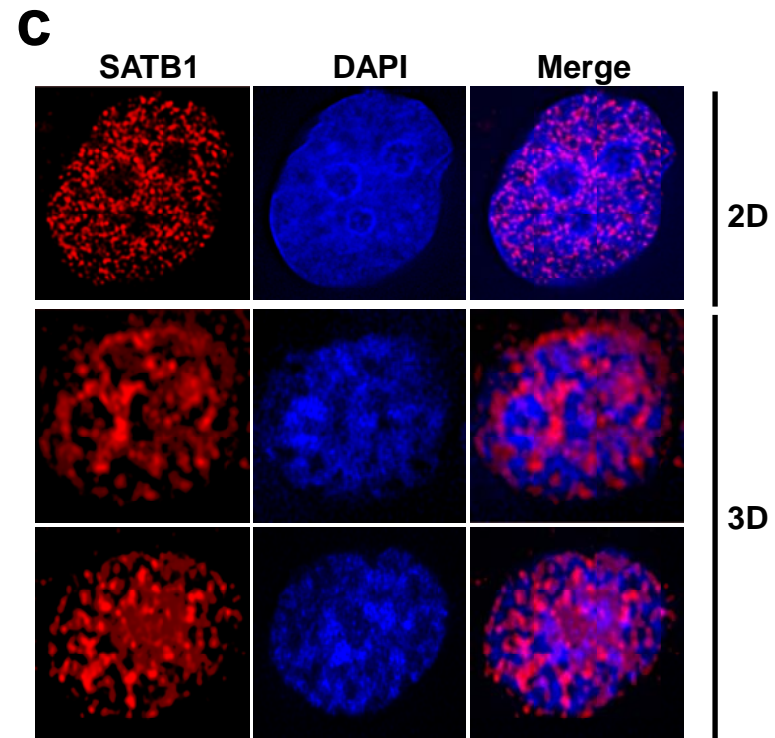
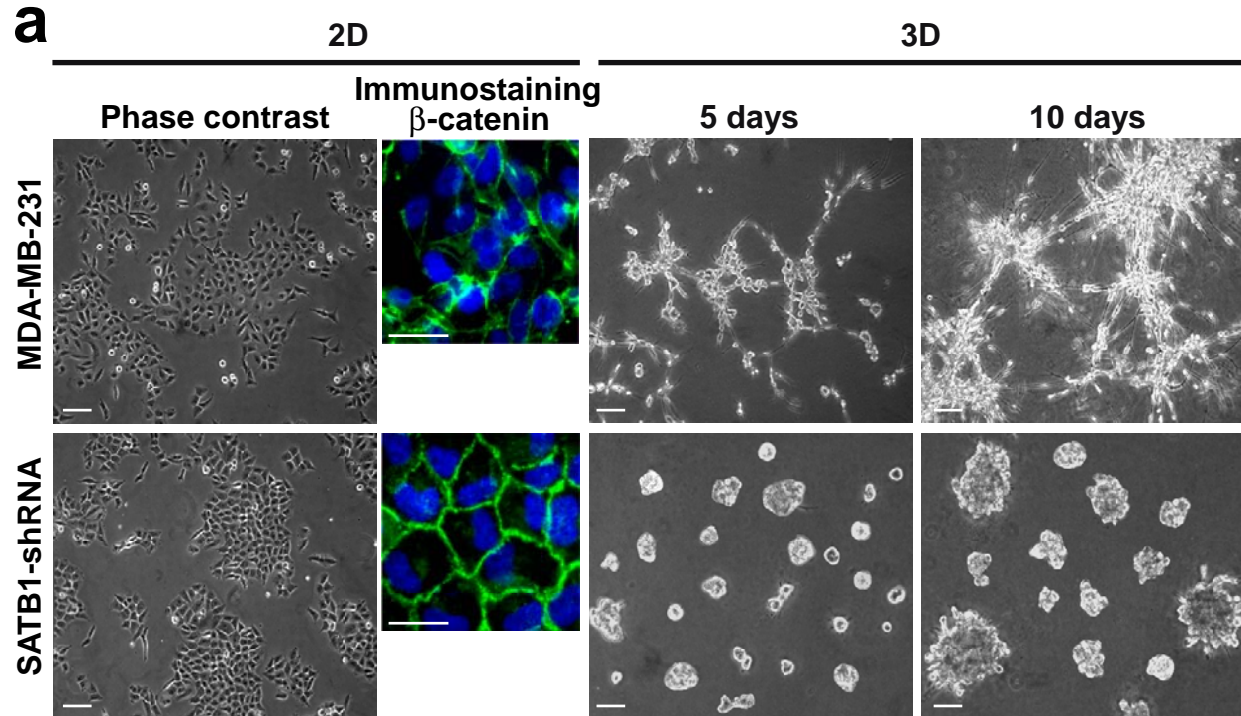
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a MDA-MB-231

Control shRNA



SATB1-shRNA1



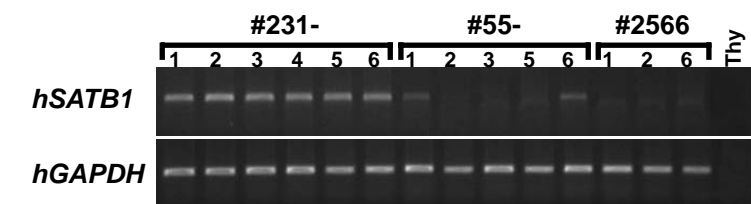
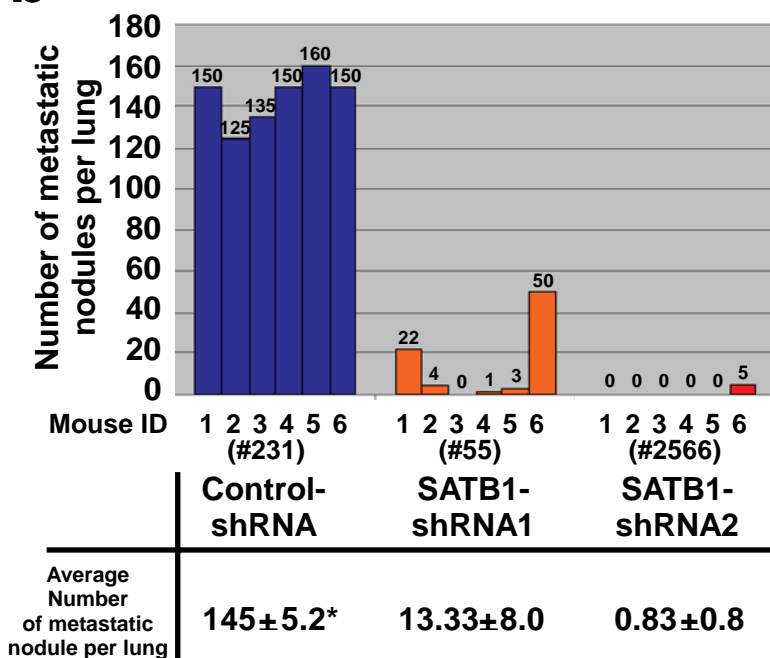
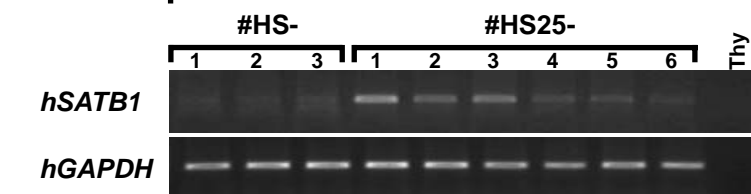
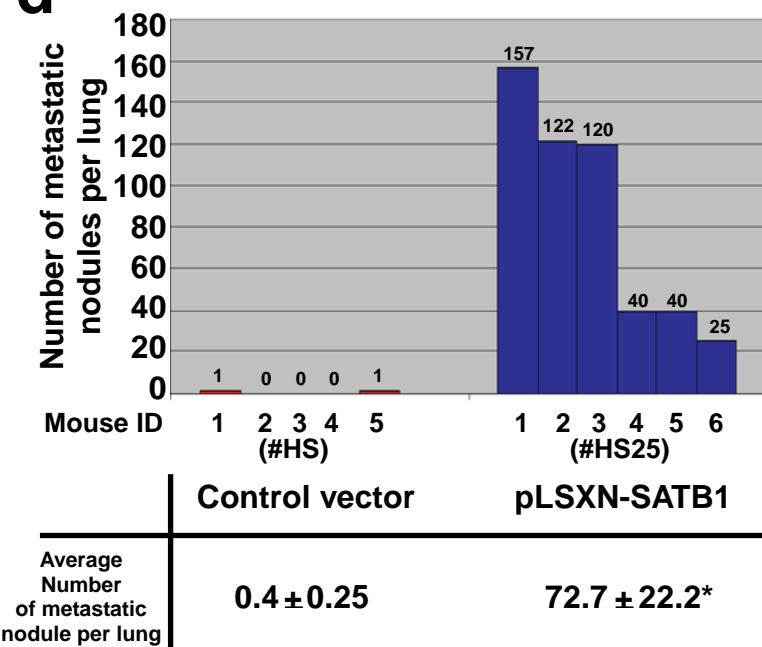
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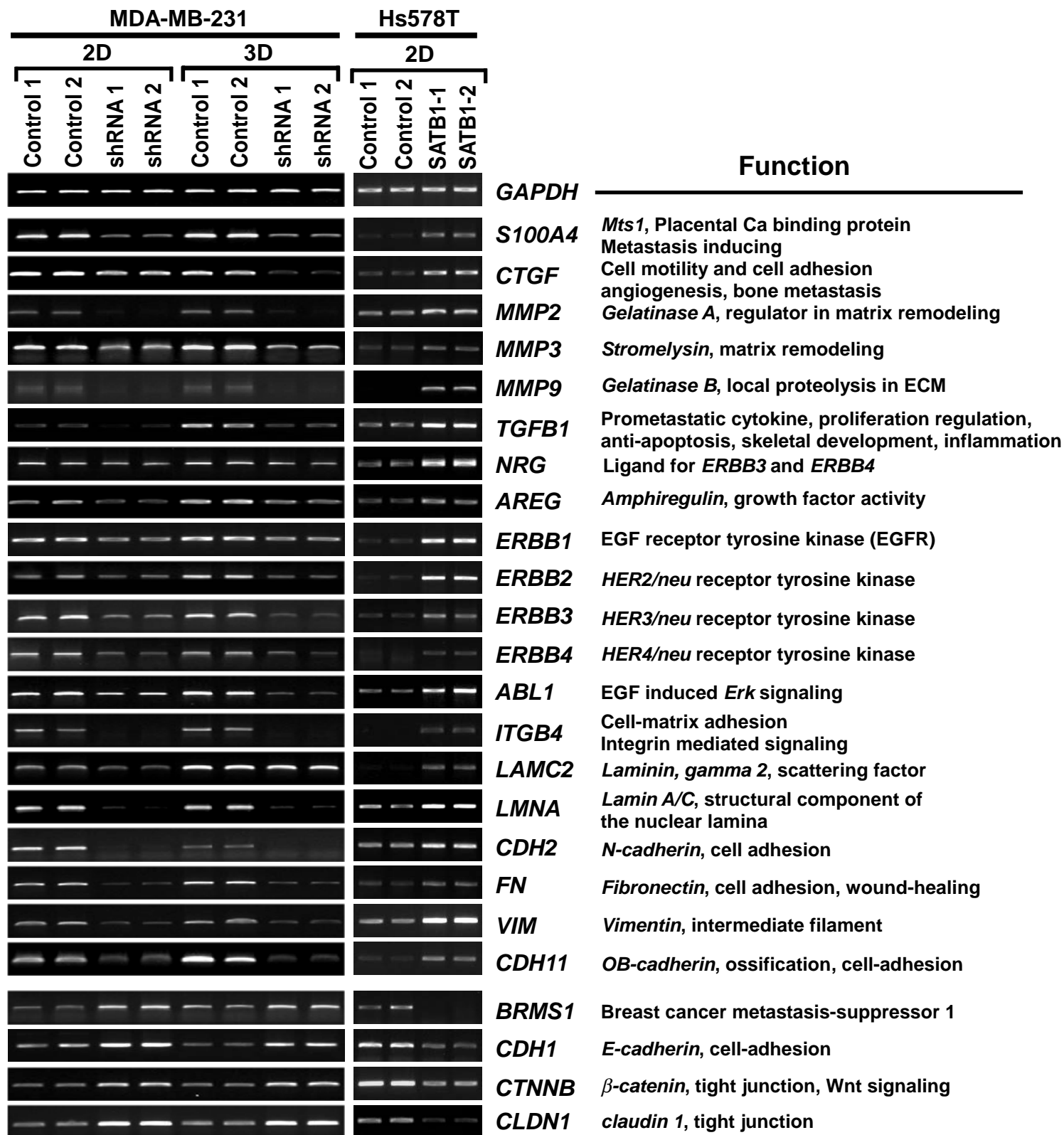
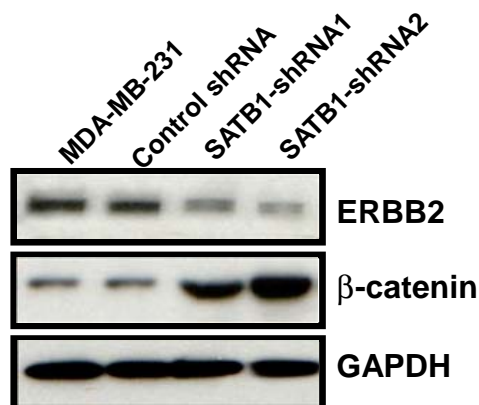
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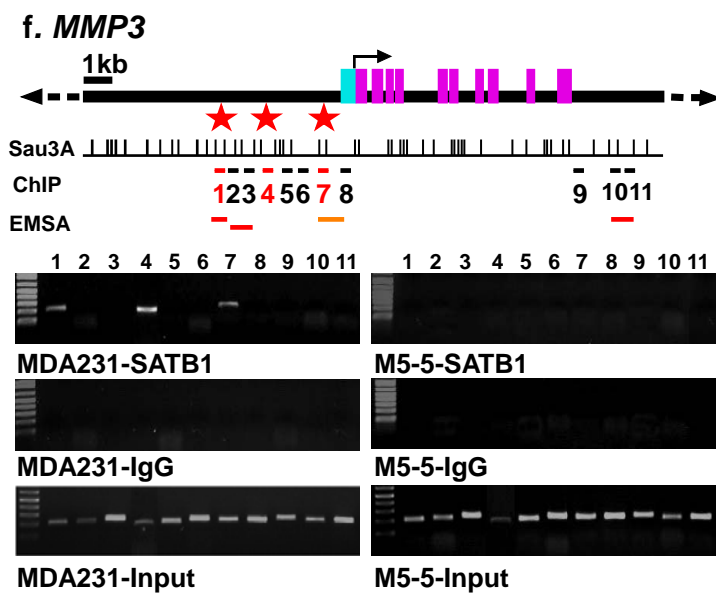
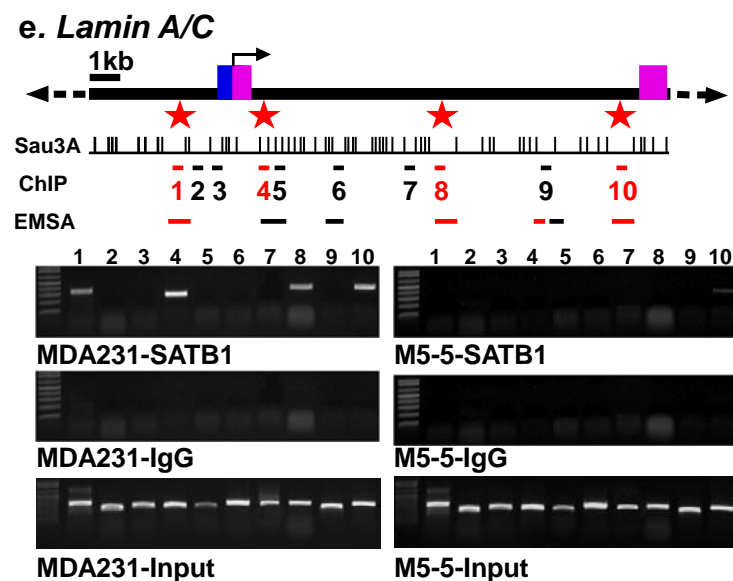
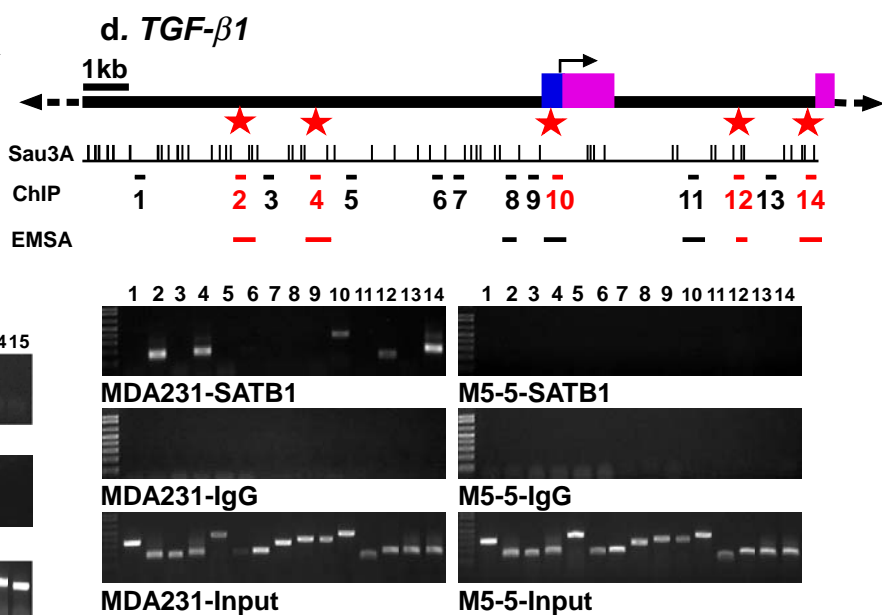
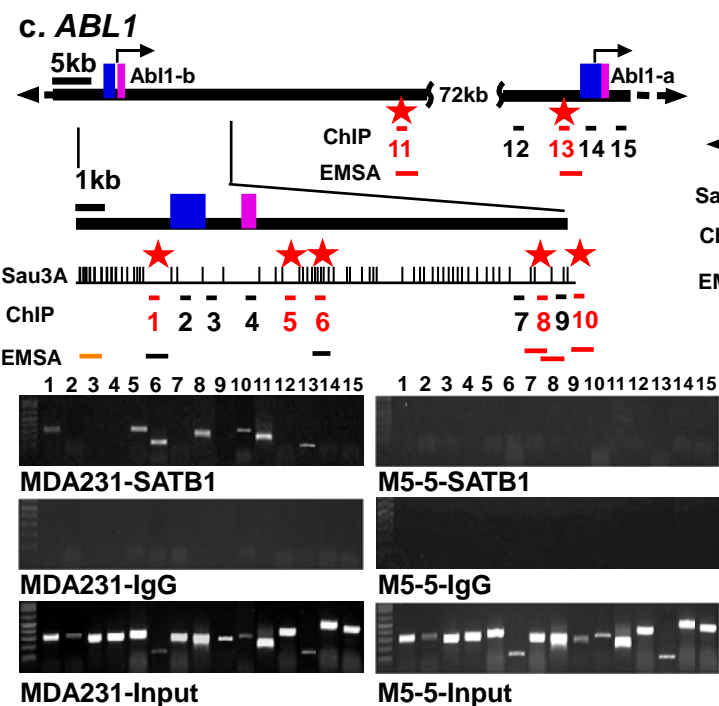
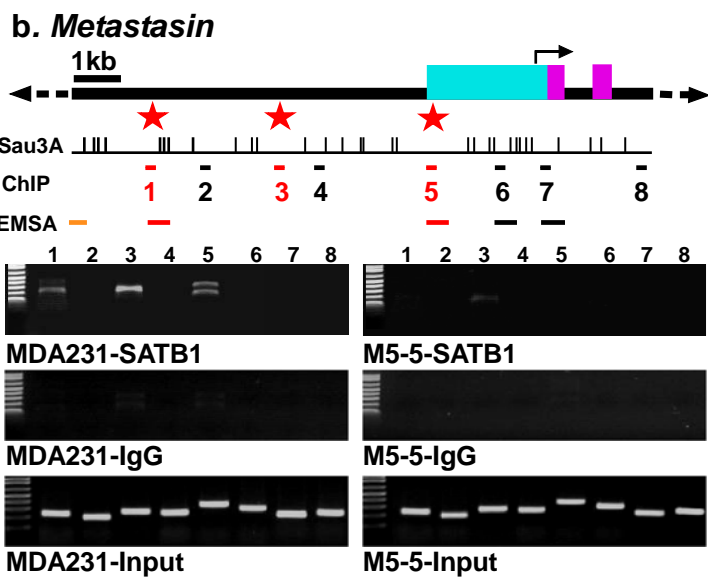
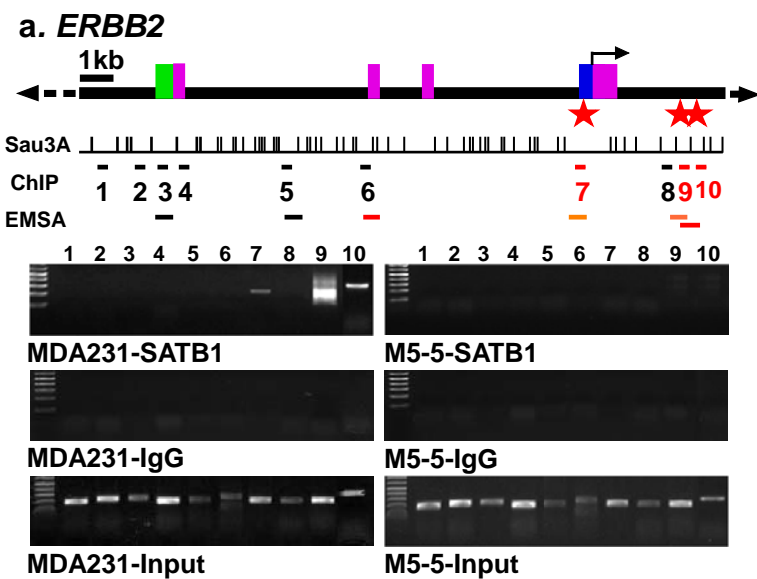
Control vector

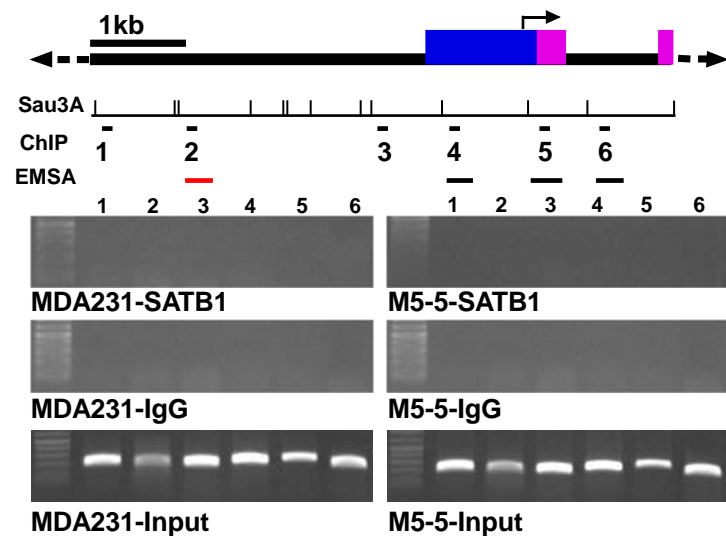
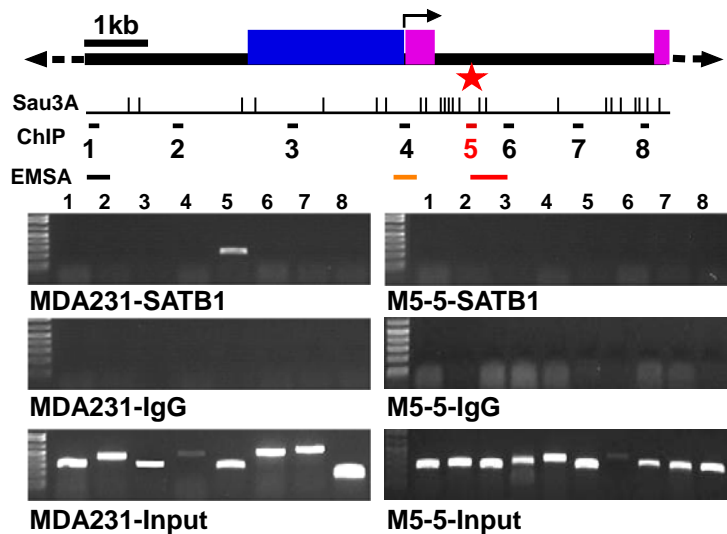
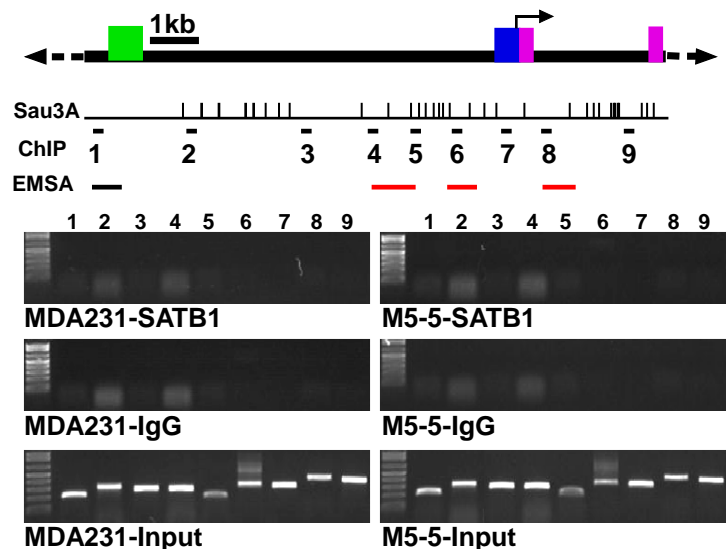
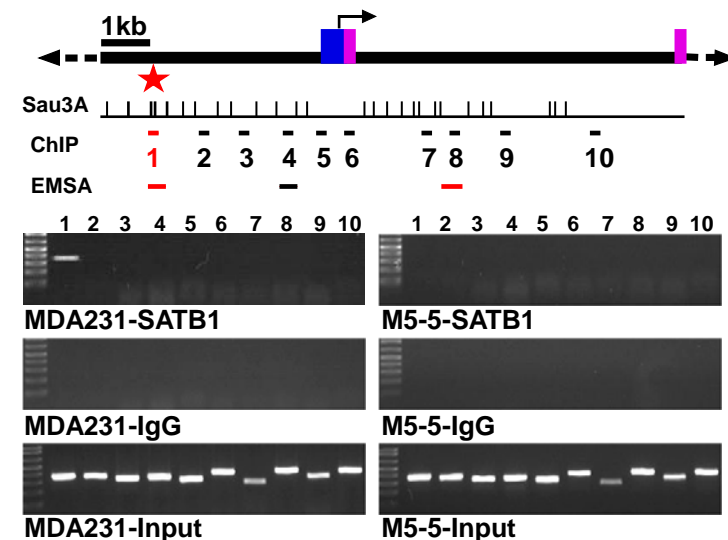


pLSXN-SATB1

**b****d**

a**b**

a

b**a. GAPDH****b. NRP1****c. TIMP1****d. ITGA5****e. ITGB5**