Epigenetic silencing of *SOD2* by histone modifications in human breast cancer cells

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Abstract

Many breast cancer cells typically exhibit lower expression of manganese superoxide dismutase (MnSOD) compared to the normal cells from which they arise. This decrease can often be attributed to a defect in the transcription of *SOD2*, the gene encoding MnSOD; however, the mechanism responsible for this change remains unclear. Here, we describe how altered histone modifications and a repressive chromatin structure constitute an epigenetic process to down regulate *SOD2* in human breast carcinoma cell lines. Utilizing chromatin immunoprecipitation (ChIP) we observed decreased levels of dimethyl H3K4 and acetylated H3K9 at key regulatory elements of the *SOD2* gene. Consistent with these results, we show that loss of these histone modifications creates a repressive chromatin structure at *SOD2*. Transcription factor ChIP experiments revealed that this repressive chromatin structure influences the binding of SP-1, AP-1, and NFκB to *SOD2* regulatory cis-elements *in vivo*. Lastly, we show that treatment with the histone deacetylase inhibitors trichostatin A and sodium butyrate can reactivate *SOD2* expression in breast cancer cell lines. Taken together, these results indicate that epigenetic silencing of *SOD2* could be facilitated by changes in histone modifications and represent one mechanism leading to the altered expression of MnSOD observed in many breast cancers.

Keywords

methylation; chromatin; mammary; carcinoma; antioxidant

Introduction

Tumor cells are characterized by aberrant production of reactive oxygen species and an atypical redox state [1]. This has led to the hypothesis that reactive oxygen species may be causally involved in carcinogenesis. Changes in free radical biology in tumor cells can be linked to the altered expression pattern of antioxidant enzymes like MnSOD. Manganese superoxide dismutase (MnSOD) is a mitochondrial matrix enzyme encoded by the nuclear gene *SOD2*. Cancers originating from diverse tissue types often exhibit decreased MnSOD activity compared to the tissues from which they arise. At the same time, forced MnSOD over-expression in tumor derived cell lines attenuates their malignant phenotype and lowers their

metastatic potential [2-6]. These observations suggest that MnSOD may function as a new type of tumor suppressor gene [7].

Breast cancer cells often have decreased levels of MnSOD compared to their normal counterparts, and this is generally attributed to a transcriptional defect of *SOD2* [8]. SOD2 is regulated by several transcriptional regulatory elements controlled that contain binding sites for AP-2, EGR-1, AP-1 and SP-1, NFκB and FOXO3a [9-14]. While the cis-regulatory elements bound by these transcription factors are not commonly deleted or mutated in breast cancer [15], their accessibility to binding is required for them to play an active role in *SOD2* transcription. The accessibility of DNA and transcriptional regulatory elements is actuated through epigenetic processes.

Epigenetic alterations are a means by which gene expression can be altered independent of changes in DNA sequence. In the past decade it has become abundantly clear that epigenetic changes play just as important a role as mutations in carcinogenesis. Cytosine methylation in CpG di-nucleotides is an epigenetic process that can silence gene expression. Hypermethylation of CpG di-nucleotides within the *SOD2* basal promoter has been correlated with reduced levels of MnSOD in multiple myeloma and pancreatic carcinoma [16-18]. Similar studies from our group have revealed that DNA methylation reduces MnSOD levels in immortalized fibroblasts and breast cancer cells [19,20]. However, this observation was not ubiquitous among all breast cancer cell lines with decreased expression of *SOD2*. For example, MB-231and T47D, two cell lines used in this study, are two examples of human breast carcinoma cell lines that have decreased MnSOD expression, but whose *SOD2* promoters are unmethylated (Supplemental Figure 1 and data not shown). Here, we chose to investigate whether changes in the post-translational modification of histone tails could facilitate the decreased levels of MnSOD in human breast cancer cell lines.

The post-translational acetylation and methylation of lysine residues in histone tails is another epigenetic process that can regulate gene expression [21,22]. Reduced levels of H3 histones methylated at lysine-4 and acetylated at lysine-9 within gene regulatory elements are indicative of gene silencing [23]. Previous studies have shown that MnSOD levels directly correlate with the acetylation status of histones present at *SOD2* [10,24]. Collectively these studies define the potential role for epigenetic regulation of *SOD2*.

We hypothesized that decreased levels of MnSOD in human breast cancer cell lines may be due, at least in part, to altered histone modifications and condensed chromatin structure at *SOD2*. In this study we identified decreased MnSOD protein and mRNA in four breast cancer cell lines compared to the immortalized, nontumorigenic breast epithelial cell line MCF-10A. Chromatin immunoprecipitation assays determined that the decreased expression of MnSOD in breast cancer cell lines correlated with hypoacetylation of H3K9 and hypomethylation of H3K4 at seven transcriptional regulatory elements within *SOD2*. Chromatin accessibility assays revealed that the chromatin structure of these seven regions is inaccessible compared to MCF-10A. Transcription factor chromatin immunoprecipitation uncovered decreased binding of NFκB, AP-1 and SP-1 at *SOD2* in breast cancer cell lines. A causal role of histone hypoacetylation in repressing MnSOD levels was determined by activating *SOD2* transcription using histone deacetylase inhibitors. Combined, these results suggest that MnSOD expression can be repressed in breast cancer by hypoacetylation and hypomethylation of histones, thus forming a closed chromatin structure at *SOD2* that inhibits transcription factor function.

Methods and Materials

Tissue culture and drug treatments

The cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and grown as previously described [20]. Cells were maintained at 37°C and 5% CO_2 during all experiments. MB-231, T47D, MCF7 and MCF7-5C cells were either left untreated, or treated with 200 ng/ml of trichostatin A, or sodium butyrate dissolved in DNMSO.

Quantitative reverse transcription real-time PCR analysis

Real-time PCR analysis of *SOD2* expression was carried out as previously described [20]. Analysis of SOD2 transcript levels was determined using $2 \times$ SYBR real time master mix (Applied Biosystems) and primers specific to *SOD2*, or the control gene 18S. SYBR green flouresence was then monitored using an ABI 7000 Sequence Detection System (Applied Biosystems). Once reactions were complete, a threshold of amplification was selected to calculate the cycle threshold (Ct) for each sample using the ABI Sequence Detection software (Applied Biosystems). The Ct values from triplicate reactions were then averaged for *SOD2* and 18S. Relative *SOD2* expression between cell lines was then calculated by normalizing to the 18S control using the ΔΔCt method.

Western blot analysis

Total protein extracts were resolved by 4-20% SDS-polyacrylamide gels (Bio-Rad) and transferred to a nitrocellulose membrane for western analysis. After transfer was complete blots were stained with Ponceau S to ensure equal loading and transfer. Membranes were then probed with Anti-MnSOD primary antibody in PBS + 5% non-fat dry milk in PBS containing 0.1% TWEEN 20, followed by washing with PBS. Secondary anti-rabbit horseradish peroxidase antibody conjugate was then added. After subsequent washing, antigens were visualized using the ECL Plus kit (Amersham Pharmacia) and exposing the membranes to BioMax MR film (Eastman Kodak).

MnSOD activity assay

Total protein extracts were prepared as described under western blot analysis. The SOD activity gel assay was carried out as described [25].

Chromatin immunoprecipitation assay

The preparation of cells sonicated chromatin was carried out as described by Hitchler *et al.* 2006 [20]. Prepared chromatin was then mixed with antibodies specific to: di-methylH3K4, acetylated H3K9, NFκB p65, SP-1 (Upstate Biotech), AP-1 (Santa Cruz Biotechnology) and control nonspecific IgG (Sigma) and allowed to mix overnight at 4°C with agitation. Protein/ Antibody/DNA complexes were then collected using 70 µl Protien A agarose (Upstate Biotech) followed by washing and elution according to the suggested protocol. The specificity of each antibody to pull down its respective antigen was then confirmed by western blot (Supplemental Figure 2) Protein/DNA crosslinks were then reversed using 200 mM NaCl at 65°C for 4 h and DNA purified from input chromatin and immunoprecipitation reactions using the Qiagen DNeasy Kit (Qiagen) according to manufacture's suggested protocol. Purified DNA was then quantified by using a NanoDrop ND-1000 (NanoDrop). All samples were analyzed by SYBR green quantitative real time PCR with primers designed to *GAPDH* (GAPDHf: 5′-CGTCCTTGACTCCCTAGTGTCCT-3′, GAPDHr 5′-CCTACTTTCTCCCCGCTTTTTT-3′) and the seven regions of *SOD2* (Fig. 2). Amplification of the target amplicon was monitored as a function of increased SYBR green fluorescence. An analysis threshold was set and the cycle threshold (Ct) computed for each sample. The fold

enrichment of each target sequence was determined using the following formula (Fold enrichment=$2^{(Ct \text{ of Ip'ed DNA-Ct of input})}$).

Chromatin accessibility assay

Chromatin accessibility experiments were conducted as described by Rose *et al.* 2006 [26]. The accessibility indices of cis-regualtory regions were determined using the same quantitative real-time PCR primers for amplicons A1-A7 using reactions as described under ChIP experiments (Fig. 2). The accessibility index for each amplicon was determined by the following formula (Accessibility index=$2^{((Ct \text{ Dnase treated}) - (Ct \text{ Uncut}))}$).

Statistical analysis

Statistical analysis of ChIp and Chromatin accessibility was determined using a one-way ANOVA analysis with Tukey's multiple comparison post test. All analysis was done using GraphPad Prism Version 4.0 (GraphPad Software Inc).

Results

Breast cancer cell lines have decreased expression of MnSOD and SOD2 mRNA

To begin our study we analyzed MnSOD protein and SOD2 mRNA in cell lines that are devoid of aberrant DNA methylation (Supplemental Figure 1 and data not shown).

Quantitative RT-PCR demonstrated that MB-231, T47D, MCF7 and MCF7-5C breast cancer cell lines had significantly decreased *SOD2* mRNA, and were approximately one-third of the steady state levels of *SOD2* mRNA compared to nontumorigenic MCF-10A (Fig. 1A). To query how this change in *SOD2* expression might influence free radical biology in these cell lines MnSOD protein levels and activity were also analyzed. MnSOD protein and activity was determined to be decreased in MB-231, T47D, and MCF7 breast cell lines (Fig. 1B). These results are consistent with our quantitative RT-PCR studies and confirm the previously reported observation that breast cancer cell lines generally have decreased MnSOD mRNA, protein, and activity [2].

Breast cancer cell lines have a repressive histone code at SOD2

Different histone modifications at a gene can collectively form a "histone code" that can tightly regulate gene expression [21,22]. Others have demonstrated that transcription of MnSOD directly correlates with its H3K9 acetylation after induction of *SOD2* [10]. We sought to investigate whether the decreased levels of MnSOD in our breast cancer cell lines could be attributed to loss of two histone modifications associated with active transcription, H3K4 dimethylation and H3K9 acetylation, at *SOD2* [23]. To meet this objective we chose to compare and contrast the levels of these two modifications at transcriptional regulatory elements within *SOD2* between breast cancer cell lines and MCF-10A using chromatin immunoprecipitation (ChIP). Amplicon 1 (A1) is located in the extreme 5′ region of *SOD2* and is devoid of known transcriptional regulatory elements. The other six regions were chosen because they have established roles in regulating *SOD2* (Fig. 2). Enrichment of dimethyl H3K4 was significantly different between transcriptionally inactive A1 and the other six amplicons (A2-A7) in MCF-10A and suggests that H3K4 methylation may have a role in controlling *SOD2* (Fig. 3). Similar analysis in MB-231, T47D, and MCF7 and MCF7-5C showed the level of dimethyl H3K4 enrichment was significantly different from MCF-10A at amplicons A2, A3, A4, and A7, but not at A1, A5, A6 or *GAPDH* (Fig. 3) (ANOVA $p < 0.05$, for individual statistical analysis see Supplemental Table S1). Similar quantitative ChIP analysis of H3K9 acetylation in MCF-10A again revealed that this active mark of transcription was statistically different at A1 compared to amplicons A2-A7. We were also able to determine that acetylated H3K9

enrichment was significantly different between respective amplicons in MCF-10A and MB-231, T47D, MCF7 and MCF7-5C breast cancer cell lines (Fig. 4). No enrichment of these seven amplicons was detected in control IgG immunoprecipitations (data not shown). Taken together, these tight associations give insight into the influence exerted by histone modifications on transcriptional regulatory elements of *SOD2*. Our analysis further suggests that *SOD2* may be transcriptionally repressed in cancer cells by a repressive histone code.

Breast cancer cell lines have condensed chromatin structure at SOD2

To establish whether the decrease in H3K4 dimethylation and H3K9 acetylation might form a closed chromatin structure at *SOD2*, we queried its chromatin accessibility using DNase I hypersensitivity. Chromatin accessibility at the six transcriptional elements of *SOD2* were in good agreement with their level of histone acetylation in MCF-10A and statically different from amplicon A1 (Fig. 5) (p < 0.05 ANOVA, for individual statistical analysis see Supplemental Table S1). Comparing the average accessibility of these six regions in MCF-10A with MB-231, T47D, MCF7 and MCF7-5C revealed that breast cancer cell had considerably condensed chromatin at *SOD2* relative to breast epithelial cells. Overall our findings revealed that an inaccessible chromatin structure at *SOD2* could be decreasing the level of MnSOD in these cell lines.

The binding of transcription factors at SOD2 is altered in breast cancer cell lines in vivo

The existence of repressive histone modifications and chromatin structure can impede transcription by limiting transcription factor binding. Overall, the pattern of SP-1 AP-1 and NFκB binding at *SOD2* is in good agreement with previous reports (Fig 6A-B). SP-1 binding was limited to the basal promoter region (amplicons 4 and 5) and was decreased in breast cancer cell lines compared to MCF-10A (Fig. 6A top panel). Enrichment of amplicons 2, 4 and 5 was observed in AP-1 immunoprecipitations; however only amplicon 5 enrichment was decreased in breast cancer cell lines (Fig. 6A, middle panel). Breast cancer cell lines also exhibited decreased levels of NFκB binding at amplicons 2, 3 and 7 (Fig. 6A, bottom panel). Taken together these results support the overall hypothesis that an altered chromatin environment can inhibit the transcription of *SOD2*.

Histone deacetylase inhibitors increase SOD2 transcription

Epigenetic repression by histone hypoacetylation can be reversed through the pharmacological inhibition of histone deacetylases (HDACs). To test if histone hypoacetylation was causally responsible for repressing *SOD2* in breast cancer we treated our panel of cell lines with trichostatin A (TSA) and sodium butyrate. Treatment of MB-231, MCF7 and MCF7-5C with 200 ng/ml TSA for 12 hours moderately increased their steady state levels of *SOD2* mRNA, no response was elicited from T47D (Fig. 7). However, treatment with 5 mM sodium butyrate did increase *SOD2* expression in MB-231, MCF7, and T47D. Moreover, such treatment increased the chromatin accessibility index of amplicon 4 of *SOD2* in MB-231 and MCF7-5C from 4-6 in untreated controls (Fig. 5) to 23-25 in TSA treated cells, consistent with chromatin remodeling to a more transcriptionally accessible structure. Together, these data suggest that histone hypoacetylation is, at least in part, causally responsible for repressing *SOD2* expression in breast cancer.

Discussion

SOD2 expression is often attenuated in breast cancer, and can usually be attributed to a defect in its transcription [8]. The *SOD2* gene is not commonly deleted or mutated in breast cancer, thus the mechanism(s) by which MnSOD levels become decreased in breast cancer remains elusive [15]. Previous studies have described the repression of *SOD2* by DNA methylation [16-20]. We sought to investigate if changes in histone modifications and chromatin structure

could constitute another means by which epigenetic processes could decrease *SOD2* expression in human breast carcinoma cells.

Epigenetic alterations are just as capable as mutations at changing the expression of tumor suppressor genes and metastasis suppressors. Mutations in the basal promoter of *SOD2* have been shown to alter transcription factor function and decrease MnSOD levels in colon cancer, glioblastoma, and fibrosarcoma [27]. At this time no such mutations have been identified at *SOD2* in breast cancer. Transcriptional repression by epigenetic processes can occur without mutation. *SOD2* contains a large CpG island, which makes it a likely candidate for repression by DNA methylation. DNA methylation has been shown to decrease MnSOD levels in immortalized fibroblasts, multiple myeloma, and pancreatic cancer [18,28,29]. We have recently reported that *SOD2* can become aberrantly hypermethylated at CpGs in breast cancer cells with decreased levels of MnSOD [20]. DNA methylation and histone acetylation often collaborate to repress gene expression [30,31]. However, we did not detect DNA methylation at *SOD2* in the breast cancer cell lines used in this study (Supplemental Figure 1 and data not shown).

The pattern of histone modifications changes during carcinogenesis [32]. Hypoacetylation of H3K9 and hypomethylation of H3K4 can both silence gene expression independent of DNA methylation [23]. When MnSOD expression is induced, histones associated with *SOD2* become hyperacetylated [24,33]. Here we have investigated the decreased expression of *SOD2* in breast cancer. We compared and contrasted the level and placement of acetylated H3K9 and methylated H3K4 at *SOD2* between breast cancer cell lines and immortalized epithelial cells. These results demonstrate that breast cancer cells have significantly lower levels of both acetylated H3K9 and methylated H3K4 at *SOD2* which is consistent with their decreased level of MnSOD.

Histone acetylation often coincides with an accessible chromatin structure and active transcription. When chromatin is accessible, transcription factors have admittance to their recognition sites. By measuring chromatin accessibility, we found that chromatin at *SOD2* in breast cancer cells is in a closed state that correlates with their level of acetylated H3K9 histones. We also determined that the level of accessibility differs between discrete transcriptional regulatory elements within *SOD2*. For example, accessibility and histone acetylation are lowest at amplicons A2 and A5 in breast cancer cells. Our transcription factor ChIP experiments concluded that AP-1 and NFκB binding was also significantly reduced at these regions. Others have demonstrated that transcriptional activation of *SOD2* by NFκB can affect histone acetylation, chromatin structure and SP-1 binding at the basal promoter [33]. SP-1 binding at the basal promoter is not a prerequisite for *SOD2* expression in mouse embryonic fibroblasts. However, SP-1 is required to mediate the transcriptional activation of *SOD2* by the intronic enhancer and other 5′ regulatory elements. Based on this knowledge, it seems likely that epigenetic inactivation of these other regulatory elements might lead to the decreased histone acetylation, methylation chromatin accessibility and decreased SP-1 binding we observed at the basal promoter (amplicon A5) of breast cancer cells.

Epigenetic silencing is perpetuated by the activity of enzymes that add and remove epigenetic marks. The activity of these enzymes can be interrupted by treatment with pharmacological inhibitors. Many of these inhibitors have been aimed at influencing the activity of HDACs. Trichostatin A and sodium butyrate are two such pharmacological agents that have been widely used to inhibit the activity of class I and class II HDACs in cancer [34]. Treatment with TSA was able to slightly increase the level of *SOD2* mRNA in MB-231, MCF7, and MCF7-5c in association with acquisition of a more accessible chromatin structure, but not in T47D. However, sodium butyrate was able to elicit a SOD2 transcription response in T47D. Reports from others have established that TSA increases MnSOD and H3K9 acetylation at *SOD2* in

mouse myoblasts [10]. Together these findings suggest that *SOD2* could be repressed in breast cancer through the activity of class I and II HDACs in the breast cancer cell lines that exhibited a response. Previous reports suggest that SP-1 can recruit HDACs to *SOD2* [10]. Our ChIP results reveal that SP-1 binding is decreased at *SOD2* in breast cancer cell lines. This implies that some as yet unknown mechanism could be recruiting HDAC activity to *SOD2*. Taken together, these results demonstrate that the *SOD2* is a potential target for pharmacological reactivation by TSA and sodium butyrate in some breast cancers.

Epigenetic regulation of *SOD2* may also be responsible for increased levels MnSOD in metastatic disease [35,36]. It is counter intuitive that mutations at *SOD2* would result in increased MnSOD. Specific histone modifications at genes are inheritable after cell division; however they can exhibit a certain plasticity. Increased H3K9 acetylation and chromatin accessibility could change at *SOD2* to increase MnSOD expression to suit the needs of tumor cells at metastatic sites. Our study has focused on examining the influence of epigenetic processes on *SOD2* in cell cells derived from human tumors. Nevertheless, when our findings are considered together with previous studies describing chromatin changes during *SOD2* transcriptional activation it seems likely that epigenetic control of SOD2 expression is consistent with all the existing data.

In conclusion, our results show changes in chromatin function at *SOD2* is associated with its decreased expression in human breast cancer. The relationship between *SOD2* expression, histone acetylation, histone methylation and chromatin accessibility are represented in the radar graphs in Fig. 8. Depicting the data in this manner illustrates the sharp contrasts of *SOD2* function in immortalized breast epithelial and breast cancer cell lines. From these observations we suggest the following model for how *SOD2* is regulated in breast cells (Fig. 9). In our model normal breast epithelial have high levels of acetylated H3K9 and H3K4 dimethylation associated with *SOD2*. In kind, the chromatin structure at *SOD2* is accessible, making cis-regulatory elements open for transcription factor binding (Figure 8a). Human breast cancer cells have decreased levels of both histone modifications at *SOD2*, which forms a closed chromatin structure which ultimately inhibits the function of AP-1, NFκB, and SP-1 (Figure 8b). The contrasting differences in the chromatin structure between immortalized breast epithelial and breast cancer cell lines suggest that epigenetic processes may, at least in part, be responsible for the altered level of *SOD2* transcription in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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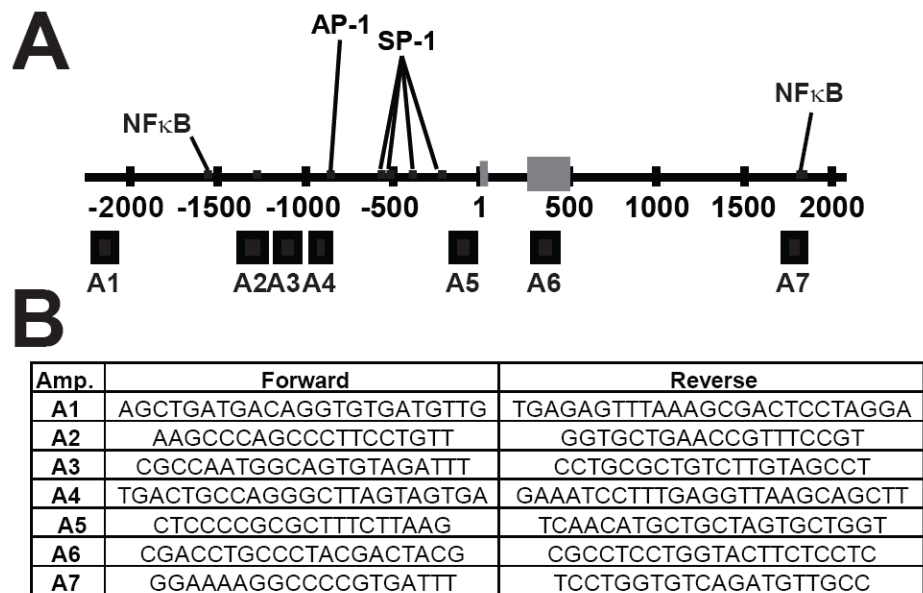
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Abbreviations

MnSOD

manganese superoxide dismutase

mRNA

messenger RNA

ChIP
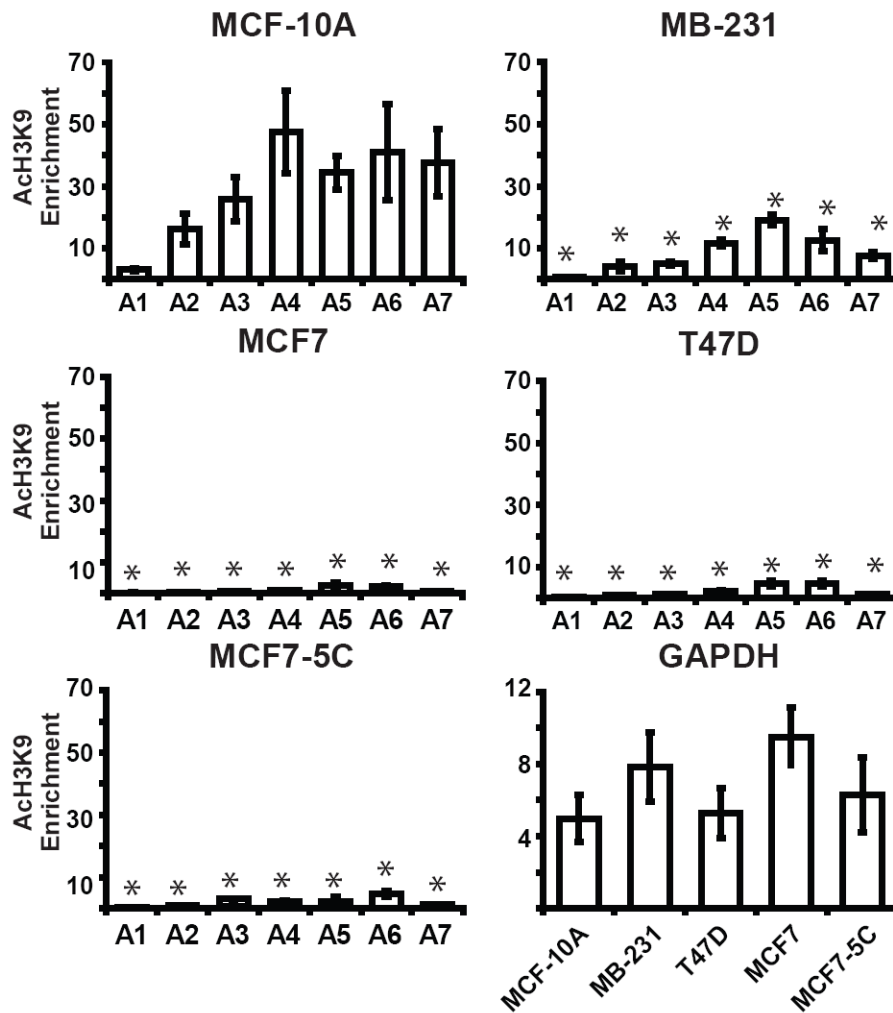
chromatin immunoprecipitation
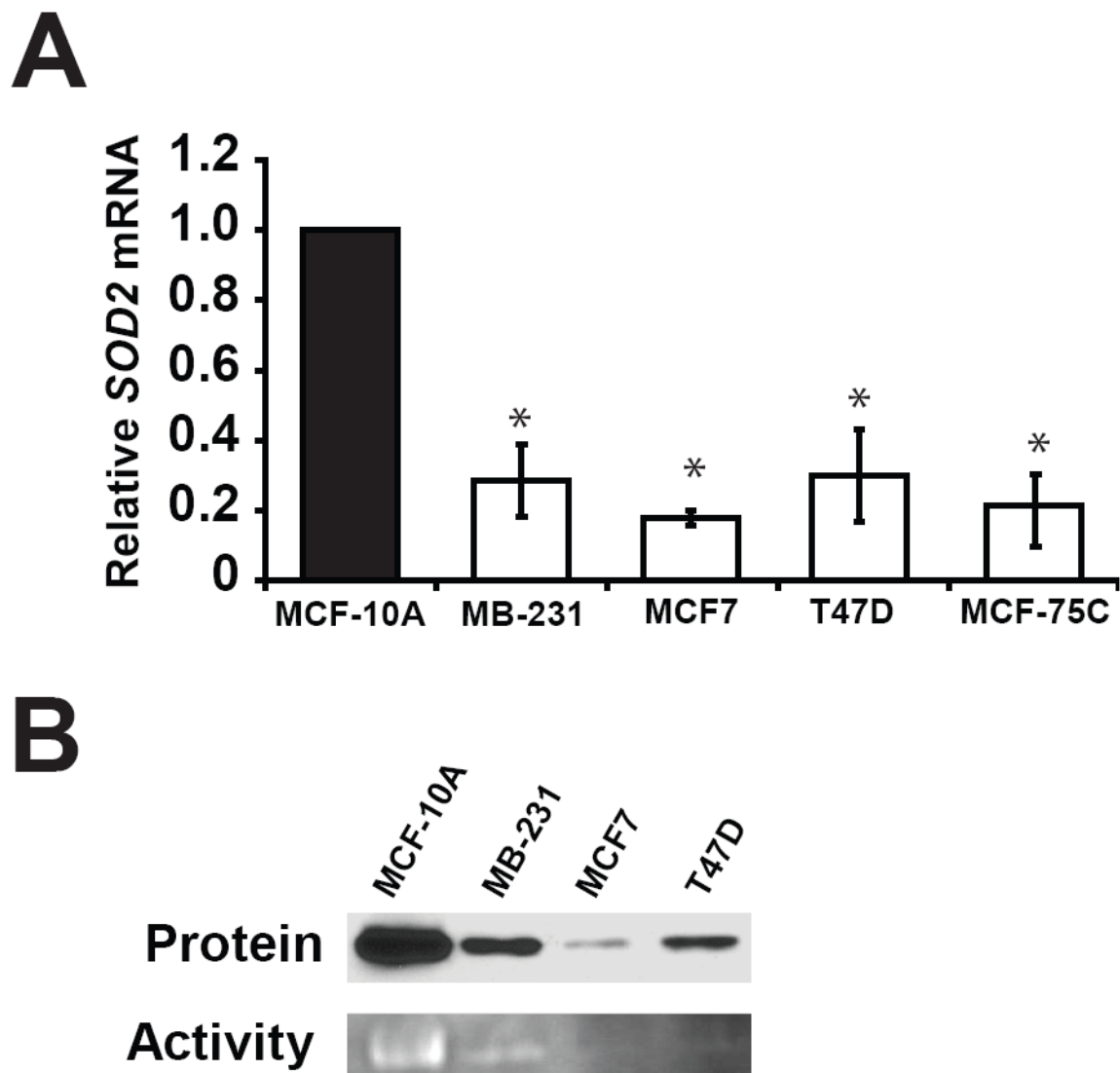
Fig. 1.
Levels of MnSOD mRNA, protein, and activity are lower in breast cancer cell lines than immortalized breast epithelial cells. (**a**) Relative *SOD2* mRNA levels in breast cell lines determined by SYBR green real-time PCR. The steady state levels of *SOD2* mRNA in the cell lines were normalized to 18S control, then compared to MCF-10A using the $\Delta\Delta$Ct method. Values shown are the average of three independent experiments ± standard error of mean (* $p < 0.05$). (**b**) The levels of MnSOD protein and activity in the model cell lines used during this study.

A

AP-1 SP-1

NFκB

NFκB

-2000 -1500 -1000 -500 1 500 1000 1500 2000

A1 A2 A3 A4 A5 A6 A7

B

Amp.	Forward	Reverse
A1	AGCTGATGACAGGTGTGATGTTG	TGAGAGTTTAAAGCGACTCCTAGGA
A2	AAGCCCAGCCCTTCCTGTT	GGTGCTGAACCGTTTCCGT
A3	CGCCAATGGCAGTGTAGATTT	CCTGCGCTGTCTTGTAGCCT
A4	TGACTGCCAGGGCTTAGTAGTGA	GAAATCCTTTGAGGTTAAGCAGCTT
A5	CTCCCCGCGCTTTCTTAAG	TCAACATGCTGCTAGTGCTGGT
A6	CGACCTGCCCTACGACTACG	CGCCTCCTGGTACTTCTCCTC
A7	GGAAAAGGCCCCGTGATTT	TCCTGGTGTCAGATGTTGCC

Fig. 2.
Locations of the seven real time PCR amplicons (A1-A7) used to analyze *SOD2* by chromatin immunoprecipitation (ChIP) and chromatin accessibility. Binding sites for AP-1, NFκB and SP-1 within *SOD2* are indicated by black lines. (**A**) The seven real time PCR amplicons used during histone and transcription factor ChIP as well as chromatin accessibility represented by black boxes below the line. Exon 1 of the *SOD2* gene are designated by grey boxes. Numbering scheme was derived from build 17 of the human genome project. (**B**) Oligonucleotide primer sequences used to analyze the seven regions of *SOD2* during chromatin immunoprecipitation and chromatin accessibility experiments.
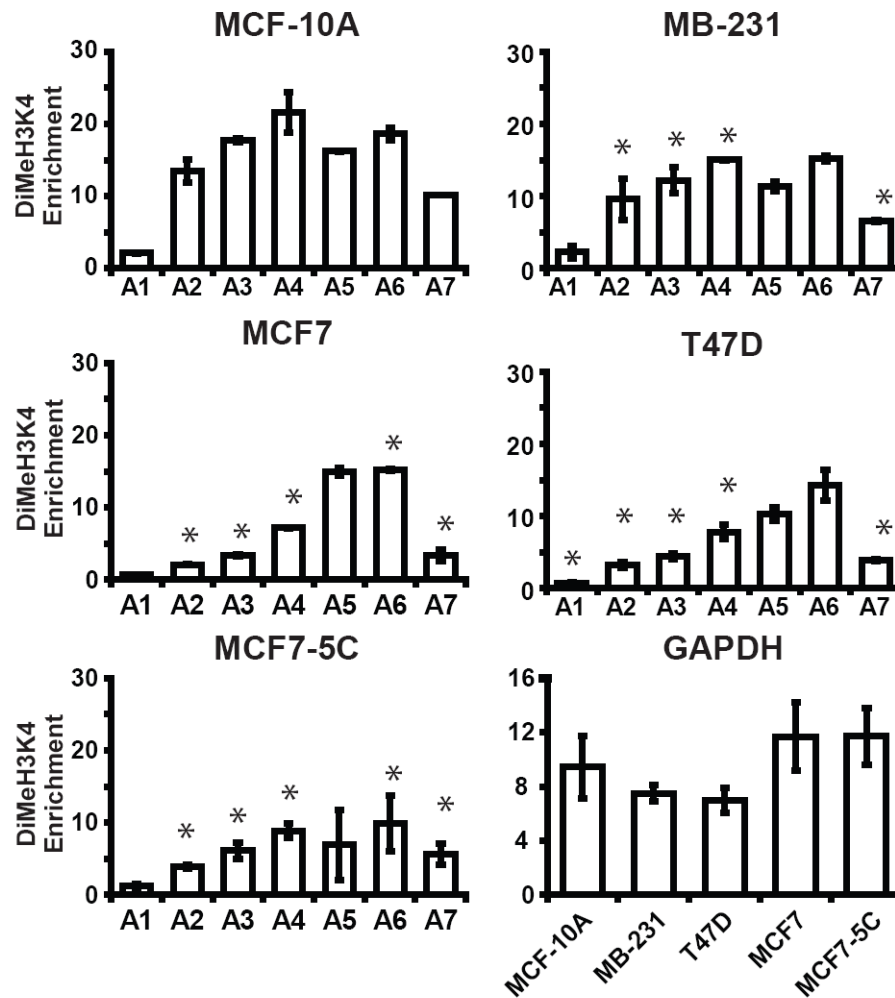
Fig. 3.
Breast cancer cells have decreased levels of dimethyl H3K4 at *SOD2* compared to immortalized breast epithelial. Methylation of H3K4 histones at seven regions (A1-A7) of the *SOD2* promoter and at *GAPDH* in breast cell lines was determined using chromatin immunoprecipitation (ChIP). Fold enrichments of dimethyl H3K4 at these regions were calculated as described in methods and materials. Values given are the averages of three independent experiments ± the standard error. (* $p < 0.05$ between MCF-10A and breast cancer cell lines at respective amplicons. For individual statistical analysis between MCF-10A and breast cancer cell lines see Supplemental Table S1).

Fig. 4.
The *SOD2* promoter of breast cancer cells is associated with hypoacetylated histones. The association of acetylated H3K9 with *SOD2* and *GAPDH* in breast cell lines was determined using chromatin immunoprecipitation (ChIP). The level of acetylated H3K9 at seven regions (A1-A7) of the *SOD2* and *GAPDH* promoters in breast cell lines were calculated as described in methods and materials. Values given are the averages of three independent experiments ± the standard error. (* $p < 0.05$ between MCF-10A and breast cancer cell lines at respective amplicons. For individual statistical analysis between MCF-10A and breast cancer cell lines see Supplemental Table S2).
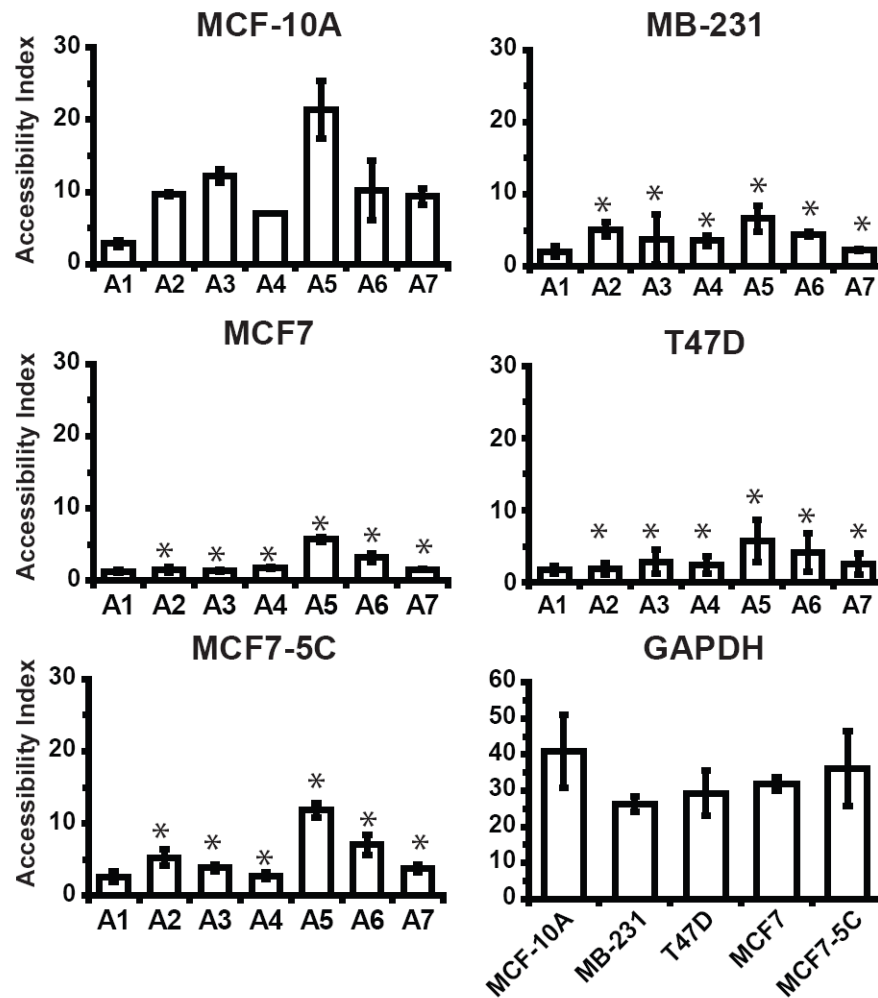
Fig. 5.
The *SOD2* gene of breast cancer cell lines is less accessible than immortalized breast epithelial cells. Chromatin accessibility of the seven regions (A1-A7) of the *SOD2* promoter and *GAPDH* was determined by DNAse I hypersensitivity. Accessibility index of each region was determined using SYBR green real-time PCR. Accessibility index for each amplicon was then as described in methods and materials. Values given are the averages of three independent experiments ± the standard error. (* p < 0.05 between MCF-10A and breast cancer cell lines at respective amplicons. For individual statistical analysis between MCF-10A and breast cancer cell lines see Supplemental Table S3).

A
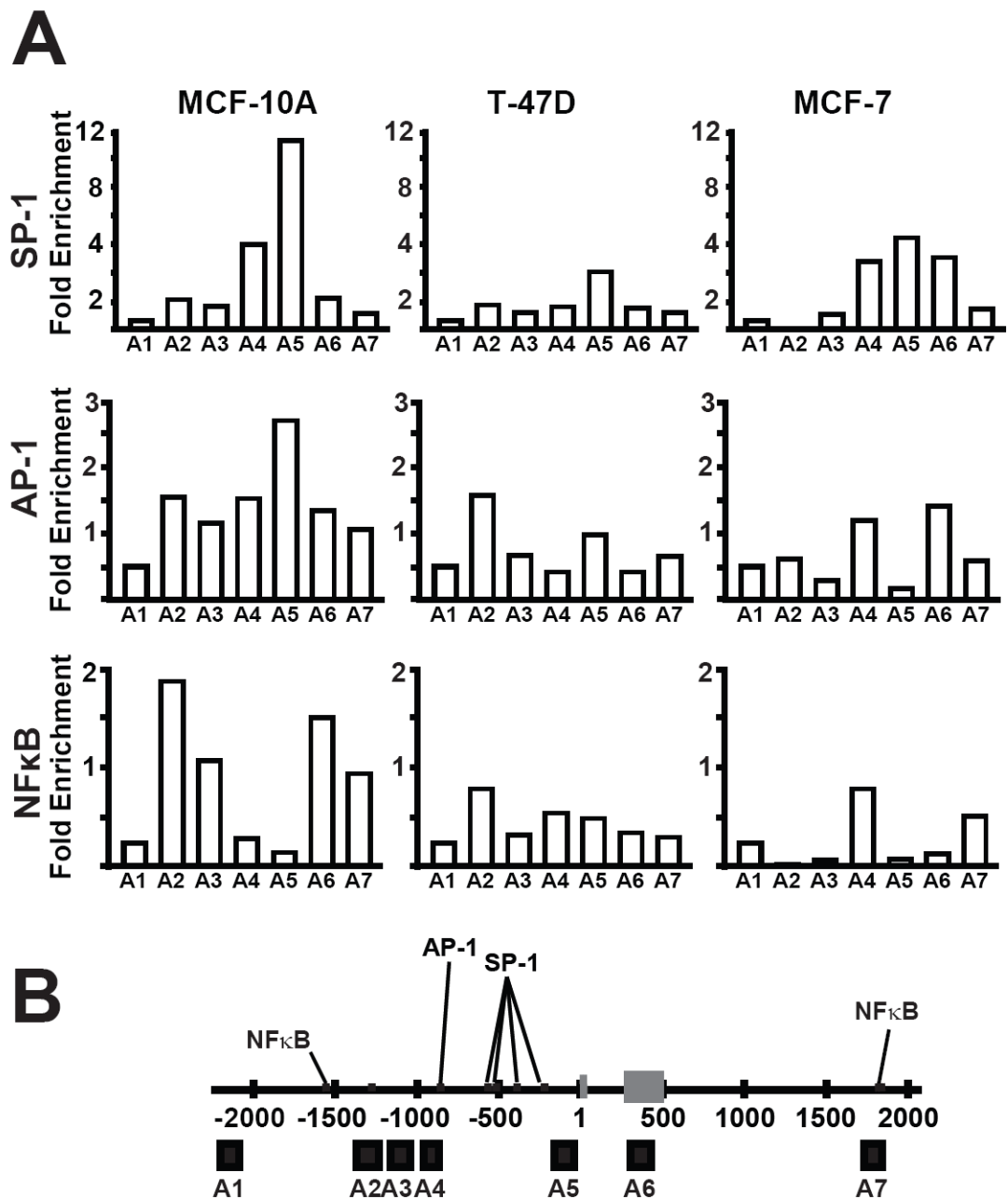
Fig. 6.
Binding of AP-1, SP-1 and NFκB are decreased at *SOD2* in breast cancer cell lines. The placement and level of SP-1, NFκB, and AP-1 binding at *SOD2* was determined using (ChIP). Fold enrichments of these transcription factors *SOD2 was* calculated as described under methods and materials.
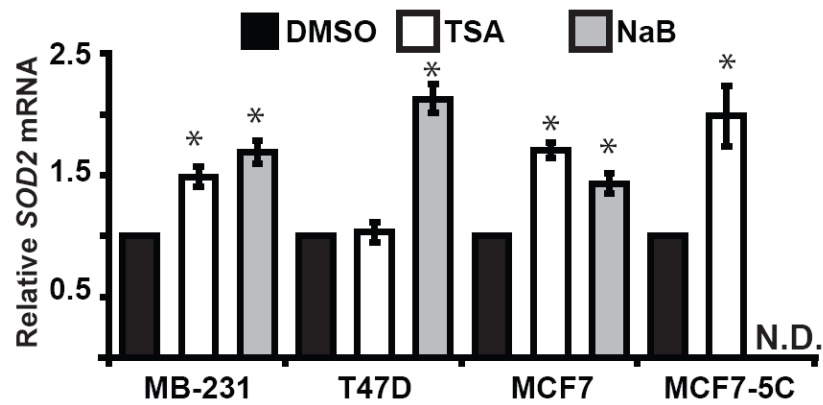
Fig. 7.
The histone deacetylase inhibitors trichostatin A (TSA) and sodium butyrate increased *SOD2* expression in breast cancer cell lines. MB-231, T47D, MCF7 and MCF7-5C breast cancer cell lines were treated 200 ng/ml trichostatin A or 5 mM sodium butyrate for 12 hours. Total RNA was then harvest and subjected to quantitative real time RT-PCR analysis to determine changes in *SOD2* expression. The levels of *SOD2* mRNA were normalized using *18S* rRNA. The relative level of *SOD2* mRNA in each sample were determined by comparing TSA and sodium butyrate treated cells with their matched DMSO controls using the $\Delta\Delta$Ct method. Values shown are the average of three independent experiments $\pm$ stand are error of mean (* $p < 0.5$ versus untreated control). N.D.; not determined.
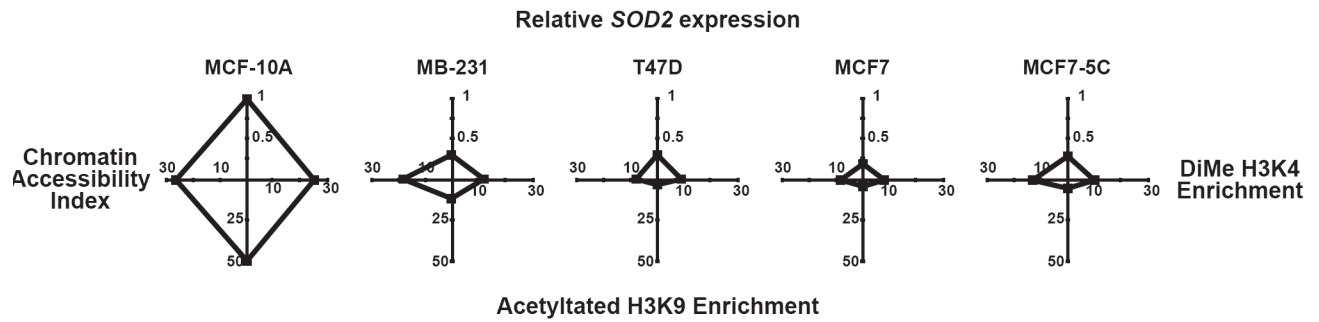
Fig. 8.
Changes in MnSOD expression correlate with chromatin function at *SOD2*. The relationship between *SOD2* expression and the chromatin parameters in breast cells represented as radar graphs. The average values of relative SOD2 mRNA (north axis) were plotted against enrichment of H3K4 methylation at amplicon 4 (east axis), enrichment of acetylated H3K9 at amplicon 4 (south axis) and chromatin accessibility (west axis). These graphs contrast the differences in chromatin biology at SOD2 to clearly relate of the changes between MCF-10A and MB-231, T47D, MCF7, and MC7-5C breast cancer cell lines.
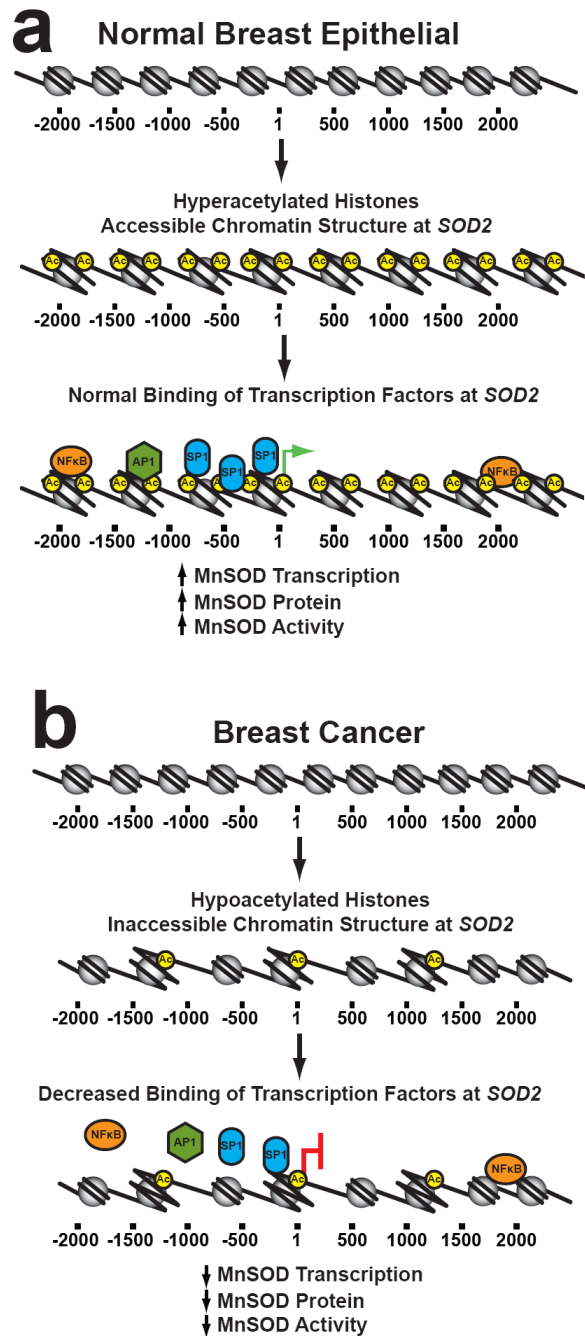
Fig. 9.
Proposed model for how changes in histone modifications alter SOD2 expression. The epigenetic state of *SOD2* when MnSOD levels are high. Hyperacetylated histones create an accessible chromatin structure at *SOD2* that allows transcription factors to bind. (**A**) When MnSOD is epigenetically repressed by histone hypoacetylation, an inaccessible chromatin structure forms at *SOD2*. This change in chromatin structure inhibits SP-1, NFκB, and AP-1 binding at *SOD2* which decreases MnSOD transcription, protein and activity. This process is reversible and increased *SOD2* expression in metastatic disease may be effected by further epigenetic alterations during progression leading to the epigenetic reactivation depicted in part (**B**).