HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2013 November 01.

Published in final edited form as:

J Invest Dermatol. 2013 May ; 133(5): 1294–1301. doi:10.1038/jid.2012.487.

CtBP1 is expressed in melanoma and represses the transcription of *p16INK4a* and *Brca1*

Hui Deng[1,2,4,#], **Jing Liu**[1,#], **Yu Deng**[1,#], **Gangwen Han**[2], **Yiqun G. Shellman**[1], **Steven Robinson**[3], **John Tentler**[3], **William Robinson**[3], **David A. Norris**[1], **Xiao-Jing Wang**[2,*], and **Qinghong Zhang**[1,2,*]

[1]Department of Dermatology, University of Colorado, Denver, Aurora, CO 80045

[2]Department of Pathology, University of Colorado, Denver, Aurora, CO 80045

[3]Department of Medicine, University of Colorado, Denver, Aurora, CO 80045

[4]Department of Dermatology, The Sixth People's Hospital of Shanghai, Shanghai Jiaotong University, Yishan Road 600, Shanghai, 200211, China

Abstract

Carboxyl-terminal binding protein 1 (CtBP1) has been shown to suppress the transcription of several tumor suppressors *in vitro*. Paradoxically, a previous report showed that CtBP1 mRNA was down-regulated in melanoma. Using immunostaining, we found that a large percentage of human melanomas were positive for CtBP1 protein. Further, we demonstrated that CtBP1 expression in melanoma cells contributes to cell proliferation and genome instability, two aspects promoting melanoma initiation and progression. *Breast Cancer Susceptibility Gene 1(Brca1)*, a core protein in DNA damage repair, was repressed by CtBP1 in melanoma cells. Consistently, Brca1 loss was found in human malignant melanoma tissues inversely correlated with CtBP1 expression levels. Additionally, the inhibitor of cyclin-dependent protein kinases (CDKs), *p16INK4a*, whose loss has been related to the pathogenesis of melanoma, was repressed by CtBP1 as well. Our findings suggest an important role of CtBP1 in the transcriptional control of *p16INK4a* and *Brca1*, with CtBP1 over-expression potentially contributing to increased proliferation and DNA damage in melanoma.

Keywords

Brca1; CtBP1; p16INK4a; transcription; tumor suppressor; melanoma

*Send correspondence to: Xiao-Jing Wang, MD, PhD, Department of Pathology, University of Colorado, Denver, Aurora, CO 80045, (303)-724-3001, Fax: (303)-724-4730, xj.wang@UCDenver.edu, Qinghong Zhang, PhD, Department of Dermatology, University of Colorado, Denver, Aurora, CO 80045, (303)-724-4051, Fax: (303)-724-4048, Qinghong.Zhang@UCDenver.edu.

#Co-first authors

Conflict of Interests

The authors declare no conflict of interests.

Introduction

Melanoma is the most deadly form of skin cancer (Hall et al., 1999). One-in-fifty Americans has a lifetime risk of developing melanoma. In 2009 nearly 63,000 were diagnosed with melanoma in the United States, resulting in approximately 8,650 deaths. The projected numbers by the National Cancer Institute for 2012 are even higher with 76,250 diagnosis and 9,180 deaths predicted (http://www.cancer.gov/cancertopics/types/melanoma). Identification of the molecules and pathways responsible for melanoma is critical to the rational development of novel preventive and therapeutic strategies.

Multiple genetic events have been related to the pathogenesis of melanoma (MacKie et al., 2009; Pho et al., 2006). The *multiple tumor suppressor 1* (*CDKN2A/MTS1*) gene encodes an inhibitor of CDKs, p16INK4a, which has been localized to 9p21, a region linked to familial melanoma and homozygously deleted in many tumour cell lines and sporadic primary melanomas (Cannon-Albright et al., 1994a; Cannon-Albright et al., 1994b; Hussussian et al., 1994; Kumar et al., 1999). The DNA damage repair pathway involving *Breast Cancer Susceptibility Gene 2 (Brca2)* has been implicated in melanoma development as well (BCLC, 1999); however, to what extent the genetic alterations of *Brca2* and *Brca1* genes contribute to melanoma is controversial (Kadouri et al., 2009). Besides genetic mutation, transcriptional and translational alterations in response to environmental risk factors play important roles in melanoma development. Specifically, *p16INK4a* has been shown to be repressed by Id1 in early melanomas (Polsky et al., 2001). Whether *Brca1* expression is transcriptionally or translationally altered in melanoma has not been studied.

Carboxyl-terminal binding protein 1 (CtBP1) is a transcriptional co-repressor which often represses tumor suppressor genes. Previously, we found CtBP1 over-expression in 80% of head and neck cancers (Deng et al., 2010) and more than 90% of invasive ductal breast cancers (Deng et al., 2011). In contrast, CtBP1 mRNA has been reported to be down-regulated in melanoma (Poser et al., 2002), yet the protein level of CtBP1 has not been assessed in melanoma samples. In this report, we examined protein expression levels of this co-repressor in melanoma tissue samples and studied the potential contribution of CtBP1-mediated transcription in melanoma cell proliferation and defective DNA repair.

Results

Previous investigation has presented data to indicate that loss of CtBP1 mRNA during melanoma progression (Poser et al., 2002), but the expression of CtBP1 protein in melanoma was unknown. Therefore, we western-blotted different grades of melanoma cell lines (radial, vertical, metastasis) and normal melanocytes. CtBP1 was detected in normal melanocytes and melanoma lines, yet higher CtBP1 expression was found in metastatic melanoma lines such as A375 and WM852 cells (supplemental Fig 1). To examine the role of CtBP1 in melanoma we stained a human melanoma tissue array with an anti-CtBP1 antibody (http://www.millipore.com/catalogue/item/07-306), which recognizes the highly conserved carboxyl-terminus of CtBP1. The specificity of the anti-CtBP1 antibody was confirmed by the lack of staining in CtBP1−/− mouse embryonic fibroblasts compared to the strong nuclear signal detected by this antibody in CtBP1-positive cells (Fig. 1a). To test the

sensitivity of this antibody, we knocked down CtBP1 in the CtBP1 containing melanoma cell line A375 using two siRNAs and performed immunofluorescence staining. Positive nuclear staining was readily detected in A375 cells, while the signal was largely attenuated in the siCtBP1 treated A375 cells (Fig. 2c, 3c). We concluded this antibody can be used to assess human CtBP1 expression. Therefore, we performed CtBP1 immunohistochemistry study (IHC) on the melanoma tissue arrays (ME1003, Biomax), which contain 21 cases of melanocyte-derived nevi, 56 cases of malignant melanoma lesions, and 20 cases of metastasis. Positive nuclear CtBP1 staining was found in a large percentage of nevi, malignant melanoma, and metastasis cases (Fig. 1b). Figure 1c displays representative cases of CtBP1 staining in malignant melanoma. In contrast, CtBP1 staining was rarely detected in normal skin (supplemental Fig. 2). Further pathological study shows CtBP1 over-expression was detected in 11/21 (52%) of benign novecellular nevi and 39/49 (80%) of stage I-II malignant melanoma cases (Fig. 1b), suggesting CtBP1 over-expression is an early event in melanoma development.

CtBP1 is a transcriptional co-repressor of multiple tumor suppressors (Chinnadurai, 2009); its transcriptional regulation is context-specific and highly dependent on the presence of transcriptional repressors which directly interact with the target genes (Chinnadurai, 2002). p16INK4a is a well known tumor-suppressor for melanoma that plays an important role in cell cycle progression (Krimpenfort et al., 2001; Kumar et al., 1999; Yang et al., 2001). We asked whether CtBP1 in melanoma could also impact p16INK4a expression since it has been shown to be a CtBP1 target in fibroblasts and keratinocytes (Mroz et al., 2008). To explore the transcriptional regulation role of CtBP1 in melanoma, we performed chromatin immunoprecipitation (ChIP) assays in melanoma cell lines. CtBP1 was recruited to the *p16INK4a* promoter in WM852 (data not shown) and A375 cells (Fig. 2a). Further, we found CtBP1 binding to the *p16INK4a* promoter confers transcriptional repression of the p16INK4a gene, as CtBP1 knockdowns using two different siRNAs increased p16INK4a mRNA level in A375 cells (Fig. 2b).

To assess if CtBP1 knockdown restores p16INK4a protein expression, we performed immunofluorescent staining of p16INK4a in A375 cells and A375 cells treated with siRNAs to CtBP1 (Fig. 2c). Consistent with the mRNA increase, the nuclear p16INK4a staining was up-regulated when CtBP1 was knocked down (Fig. 2c). Since restoration of p16INK4a expression would inhibit CDKs thus decreasing cell proliferation, we examined the growth of A375 cells and A375 cells treated with siRNAs of CtBP1. Under normal conditions, A375 cells grow exponentially and double their number daily. After 48 hrs, cell numbers quadrupled; in contrast, A375 cells with CtBP1 knockdown for 48 hrs exhibited significantly reduced growth (Fig. 2d). These data suggest that CtBP1-mediated *p16INK4a* repression abrogates p16INK4a functions, thus contributing to proliferation in melanoma.

To determine if CtBP1 represses p16INK4a expression in clinical samples, we performed IHC for CtBP1 and p16INK4a using a tissue array (ME1003, Biomax), focusing on the malignant melanoma lesions (n=56) for the larger sample size compared to the nevi (n=21) and the metastatic samples (n=20) on this tissue array (Fig. 2e). Consistent with the reported p16INK4a loss in melanoma (Jonsson et al., 2010), p16INK4a loss was detected in 35/56 (62.5%) malignant melanoma samples; among them, 76.7% (33/43) cases associated with

positive CtBP1 staining versus only 15.4% (2/13) p16INK4a loss associated with negative CtBP1 staining. The inverse correlation of p16INK4a and CtBP1 (p=0.0001) in melanoma tissues is consistent with repression of the melanoma tumor suppressor p16INK4a by CtBP1.

Genomic instability is a hallmark of melanoma development and DNA damage repair defect is a major contributor. Therefore, we investigated the role of CtBP1 in melanoma DNA damage repair. Previously, we demonstrated that *Breast Cancer Susceptibility Gene 1(Brca1)* was under transcriptional control by CtBP1 in head and neck squamous cell carcinoma (Deng et al., 2010). Later, CtBP1 was found to repress *Brca1* in breast cancer cells as well (Deng et al., 2011; Di et al., 2010). Even though Brca1 mutation has not been associated with melanoma susceptibility, Brca1 down-regulation may contribute to melanoma development via decreased DNA damage repair. Hence we asked if CtBP1 represses the tumor suppressor *Brca1* in melanoma cells. First, we assessed whether CtBP1 was recruited to the *Brca1* gene to repress transcription in melanoma cells. We performed ChIP assays and found that CtBP1 bound to the *Brca1* gene promoter in WM852 (data not shown), and A375 cells (Fig. 3a). To examine if the CtBP1 binding to *Brca1* promoter confers transcriptional repression to the *Brca1* gene in melanoma cell lines, we used two different siRNAs to knockdown CtBP1 and assayed the expression of Brca1 mRNA in A375 cells. Consistent with the increased Brca1 transcription observed with siCtBP1 treatment in HNSCC cells, *Brca1* mRNA levels increased 4–5 fold when CtBP1 was abrogated in A375 cells (Fig. 3b).

To further assess if restoration of Brca1 expression by CtBP1 knockdown in A375 cells rescues Brca1 at the functional level, we examined Brca1-mediated DNA repair foci formation by immunofluorescence staining using the A375 cells and A375-siCtBP1 cells treated with mitomycin C (MMC). Both siRNAs against CtBP1 knocked down CtBP1 effectively (Fig. 3c). Brca1 translocates to sites of MMC-induced DNA damage with other members of the Fanc/Brca pathway to form DNA repair nuclear foci (D'Andrea and Grompe, 2003). Only about 10% of A375 cells were able to form Brca1 foci, whereas A375 cells with siCtBP1 knockdown for 48 h exhibited a 3-fold increase in the number of cells able to form MMC-induced DNA repair foci, from 11.6 ± 1.2 to 40.3 ± 3.1 and 35.7 ± 1.2 per 100 cells (Fig. 3c). These data suggest that CtBP1-mediated *Brca1* repression abrogates Brca1 functions and results in fewer DNA repair foci in human melanoma cells.

To further investigate the repression of *Brca1* by CtBP1, we adopted the melanoma xenograft model using A375 melanoma cells. After the melanoma xenografts were established, siRNAs against CtBP1 were delivered *in vivo* to A375 xenografts for 2 weeks. Following the knock down of CtBP1 in the xenografts, the expression of *Brca1* was upregulated (Fig. 3d). We performed comet assays using tumor cells from the xenografts. DNA breaks were significantly reduced when CtBP1 was knocked down, from 11.3 ± 2.1 to 4.3 ± 0.6 and 5.3 ± 0.6 per 100 cells (Fig. 3e).

Next, we studied the relative expression of Brca1 and CtBP1 in melanoma cases for the potential non-genetic Brca1 loss that contributes to melanoma genomic instability (Fig. 4a). Brca1 loss was detected in 33/56 (58.9%) of malignant melanoma. Moreover, we found

Brca1 loss strongly correlated with CtBP1 over-expression: 72.1% (31/43) of cases associated with a positive CtBP1 staining versus only 15.4% (2/13) Brca1 loss associated with a negative CtBP1 staining (p=0.0007, Fig, 4b). The inverse correlation of Brca1 and CtBP1 suggests an important role of CtBP1 in transcriptional control of *Brca1* in melanoma. Consistently, the DNA damage surrogate marker, pH2AX, staining in the melanoma tissue array was inversely correlated with Brca1 expression (p=0.024, Fig, 4c).

Our results provided a potential mechanistic link between CtBP1 over-expression and melanoma genomic instability. Therefore, we were prompted to investigate the role of CtBP1 in transcriptional control of *Brca1* in samples from melanoma patients. Melanoma cells isolated from three subcutaneous melanoma metastasis cases were used in the CtBP1 knockdown experiments. No Brca1 up-regulation was detected in MB1547 upon CtBP1 knockdown (Fig. 4d); however, significant increases of Brca1 mRNA was observed in MB1589 (Fig. 4e) and MB1823 (data not shown) when CtBP1 was knocked down. In light of our findings that siRNA knockdown of CtBP1 increases Brca1 expression and function to repair DNA damage these data suggest that blocking CtBP1 activity could be a potential strategy for preventing melanoma progression by increasing repair of DNA damage and thus genome stability.

Discussion

CtBP1 has been functionally linked to proliferation, anti-apoptosis, and EMT from *in vitro* studies (Grooteclaes et al., 2003; Mroz et al., 2008; Zhang et al., 2003). Our recent study in head and neck squamous cell carcinoma found CtBP1 over-expression starting at the hyperplasia stage and revealed an additional suppressive role of CtBP1 on *Brca1*, thus providing a link to a defect in DNA damage repair and genome instability (Deng et al., 2010). Here, we have demonstrated CtBP1's transcriptional regulation of *Brca1* in melanoma cells. Furthermore, Brca1 loss was detected in human melanoma samples and correlated with increased CtBP1 staining in these lesions. Similar to the results obtained using WM852 and A375 melanoma cell lines, CtBP1 knockdown upregulated Brca1 expression in two of three melanoma cases. These findings support the notion that CtBP1 plays an important role during melanoma development by dampening DNA damage repair. Our study shows that CtBP1 over-expression and Brca1 loss are detected in both melanomas and epithelial-originated cancers, *e.g.*, head and neck cancers and breast cancers, suggesting these molecular alterations are common in carcinogenesis.

Studies on the pathogenesis of melanoma have focused mainly on genetic alterations. Some studies have suggested an increase in malignant melanoma, both cutaneous and ocular, in families with mutations in *Brca2* (BCLC, 1999). This was not confirmed in a smaller Dutch study (van Asperen et al., 2005), and studies of unselected uveal melanoma cases have not shown excess rates of *Brca2* mutations (Hearle et al., 2003). A recent study also reported an absence of founder *Brca1* and *Brca2* mutations in cutaneous malignant melanoma (Kadouri et al., 2009). This paradox may be explained by the CtBP1-mediated transcriptional control of these and other tumor suppressor genes. In fact, we have detected loss of p16INK4a and Brca1 protein in human melanoma tissues in an inverse correlation with CtBP1 levels. As a well known tumor suppressor of melanoma (Krimpenfort et al., 2001; Monahan et al., 2010;

Yang et al., 2001), p16INK4a has been shown to be down-regulated in cutaneous melanoma (Hussussian et al., 1994; Jonsson et al., 2010). Our study suggests that CtBP1 over-expression may represent a critical regulator of p16INK4a levels in melanoma. On the other hand, no Brca1 alternation has been reported in melanoma despite the association between melanoma and breast cancer reported in the literature (Larson et al., 2007; Seltzer and Leachman, 2008). Our study represents to our knowledge previously unreported identification of Brca1 loss in melanoma. Brca1 plays an important function in DNA damage repair, maintaining genome stability (D'Andrea and Grompe, 2003; Mueller and Roskelley, 2003; Shen et al., 1998). Over-expression of CtBP1 in human melanoma lesions appears to decrease the expression and function of the *Brca1* gene, thus contributing to genomic instability during melanoma initiation. MC1R, MMP-8, and β-catenin have been added to the list of tumor suppressors for melanoma (Arozarena et al., 2011; Box et al., 2001; Palavalli et al., 2009). Future studies will address whether CtBP1 affects these pathways in the context of melanoma development.

A previous study (Poser et al., 2002) has suggested a loss of CtBP1 mRNA expression in melanoma samples results in upregulation of MIA (melanoma inhibitory activity). In contrast, we have found CtBP1 protein expression is positive in a large percentage of human malignant melanoma lesions (43/56 cases) and several melanoma cell lines (supplemental Fig. 1), suggesting translational or posttranslational control of CtBP1 may be affected in melanoma. Previously, tumor suppressors such as HIPK2 and Arf have been identified to regulate CtBP1 protein stability (Paliwal et al., 2006; Zhang et al., 2003). Another potential regulator of CtBP1 protein might be melanoma associated miRNAs (Pillai et al., 2007). Improper expression of miRNA genes is seen in both benign and malignant cancers. miRNA expression profiles can be used to classify solid tumors (Lu et al., 2005) and previous study has shown that miRNA expression differs between melanoma cell lines (Gaur et al., 2007). All these can lead to CtBP1 over-expression in melanoma. The resultant down-regulation of Brca1 and its subsequent defect in DNA damage repair will increase genome instability, whereas the loss of p16INK4a would release tight control of CDKs, resulting in cell cycle progression and deregulation of the oxidative stress response in melanomas (Jenkins et al., 2011). Knocking down the central regulator CtBP1 restored the expression and function of *p16INK4a* and *Brca1* genes. We speculate that down-regulating CtBP1 activity might be of preventative or therapeutic value for human melanoma treatment.

Taken together, our study highlighted the importance of the transcriptional co-repressor CtBP1 in melanoma development via suppression of tumor suppressor genes such as *Brca1* and *p16INK4a*. Further study will aim at testing the prognostic value of CtBP1 in human melanoma as well as addressing the possibility of targeting CtBP1 as a melanoma therapy, either by itself or in combination with other current treatment.

Material and Methods

Immunofluorescence and immunohistochemistry

Consecutive slides of human melanoma array ME1003 were purchased from US Biomax (Rockville, MD).These arrays are made with tissue blocks collected within 5 years specifically for making tissue microarrays. All tissue was collected under HIPPA approved

protocols, donors were informed completely and gave their consent. After surgery, tissue samples were put into formalin. This process occurs less than 10 minutes after surgery and before the fixation. Every tissue spot on array slide is individually examined by pathologists certified according to WHO published standards of diagnosis, classification and pathological grade. Every 10th section of the tissue array is stained with H&E and reviewed by two board certified pathologists to confirm the pathology diagnosis is current and matched to the adjacent serial sections. Pathological re-confirmation report is generated and a digital image is captured. Specificity of the anti-CtBP1 antibody (Millipore, Billerica, MA) was evaluated by immunofluorescence assay using the CtBP1−/− vs. the CtBP1-postive mouse embryonic fibroblasts (MEFs) (Grooteclaes et al., 2003) before utilization in human melanoma tissue arrays. To assess the correlation between CtBP1 and the tumor suppressors p16INK4a and Brca1, antibodies against p16INK4a (Santa Cruz Biotechnology, Santa Cruz, CA), Brca1 (Santa Cruz Biotechnology, Santa Cruz, CA), and CtBP1 (Millipore, Billerica, MA) were used to stain consecutive tissue sections as we previously described (Bornstein et al., 2009). Vector Red Alkaline Phosphotase Substrate Kit 1 (Vector Laboratories, Burlingame, CA) was used in the IHC development. Evaluation of CtBP1, Brca1, and p16INK4a staining of human melanoma samples was assessed by 2 independent investigators using methods described previously (Bornstein et al., 2009). Fisher's exact test is used to calculate the p value for the correlation of CtBP1 expression with Brca1 and p16INK4a levels in melanoma samples. pH2AX (Cell Signaling) staining was also performed as an indicator of DNA damage in melanoma samples.

Cell culture and assays

Human epidermal melanocytes were purchased from Invitrogen. Melanoma samples were collected under the Declaration of Helsinki guidelines and all patients gave written informed consent under a Colorado Multiple Institutional Review Board (COMIRB) approved protocol. Isolated melanoma cells were cultured briefly for CtBP1 knockdown experiments. Melanoma cell lines were maintained in RPMI1640 with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. Melanoma cells were transfected using Lipofectamine 2000, with 100 nM scrambled siRNA (control) or siRNAs targeting CtBP1 (siCtBP1) (Bergman et al., 2009; Zhang et al., 2003) and incubated at 37°C for 48 hrs. p16INK4a expression was detected by immunofluorescence staining using a p16INK4a antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (Hoot et al., 2008). MMC-induced DNA repair foci formation was assayed using a Brca1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as we previously described (Bornstein et al., 2009). DNA breaks were detected using the comet assay (Tyagi et al., 2011). For the cell growth assay, cells were collected by trypsinization and counted using hemocytometer. For *in vivo* CtBP1 knockdown (Hobel and Aigner, 2010), 100 μl of HEPES containing polyethylenimine mixed with 1 μM scrambled siRNA (control) or siRNAs targeting CtBP1 (siCtBP1-1 and siCtBP1-2) was injected to the A375 xenografts three times/week for 2 weeks after the tumors were established in nude mice. Cells were harvested to assay their CtBP1 and Brca1 expression using qRT-PCR and their DNA breaks using the comet assay.

Chromatin immunoprecipitation (ChIP) and qRT-PCR

ChIP assays were performed on melanoma cells using an anti-CtBP1 antibody as described previously (Zhang et al., 2006). Primer sets encompassing *p16INK4a* and *Brca1* promoters were used to amplify ChIP samples in qRT-PCR: AGAGCCCCCTCCGACCCTGT and GGCGTCCCCTTGCCTGGAA for *p16INK4a*, CAATCAGAGGATGGGAGGGACAGA and CAGAGCCCCGAGAGACGCTTG for *Brca1* gene; CCACTGCGTCCAGCCATTCTTGT and CTTGAGAGGCCAAGGGAGGGTAGA for non-target. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and qRT-PCR was performed as previously described (Zhang et al., 2006). An 18S probe was used as an internal control. The relative RNA expression levels were determined by normalizing to internal controls; values were calculated using the comparative Ct method. Samples were assayed in triplicate for each experiment and at least two independent experiments were performed. Data are presented as mean ± SEM (n=3) from a representative experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by grants from the NIH RO1CA87949 (to X.J.W) and RO1CA115468, DOD CA110462 and RO3DA033982 (to Q.Z.).

Abbreviations

CtBP1	Carboxyl-terminal binding protein 1
CDK	cyclin-dependent protein kinase
EMT	epithelial-mesenchymal transition
ChIP	chromatin immunoprecipitation
IHC	immunohistochemistry
MMC	mitomycin C

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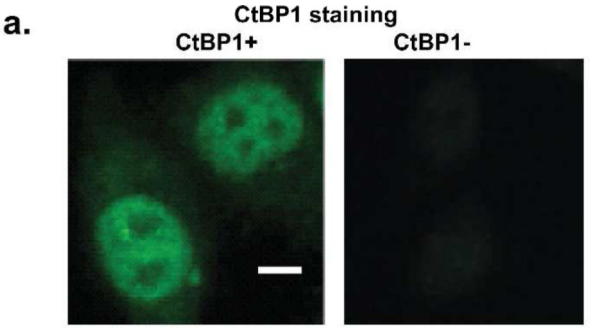


b.

Number of CtBP1 Positive Cases/Total Cases	
Benign Novecellular Nevi	11/21 (52%)
Malignant Melanoma	43/56 (77%)
Metastasis	14/20 (70%)
Tumor Stage	
Stage I, II	39/49 (80%)
Stage III, IV	4/7 (57%)
Node Status	
N0	39/49 (80%)
N+	4/7 (57%)
Metastasis Status	
M0	41/54 (76%)
M+	2/2 (100%)

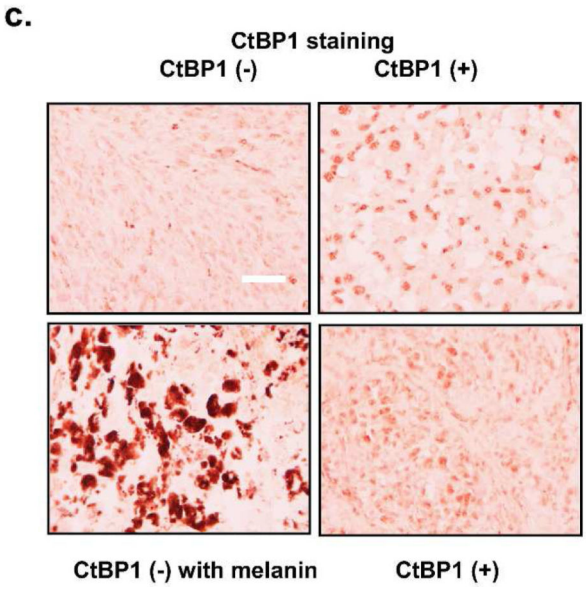
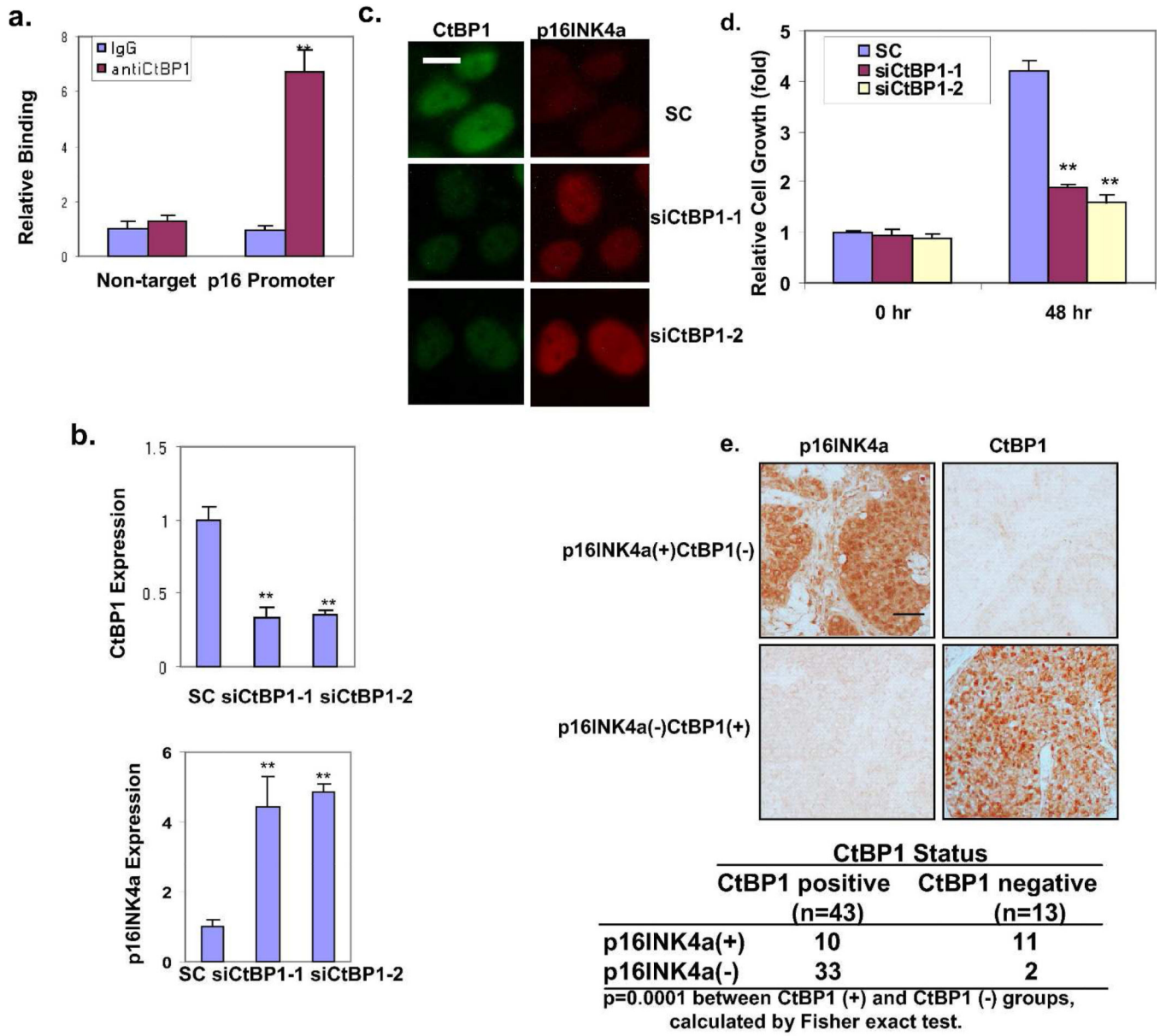


Figure 1. CtBP1 over-expression in human melanoma tissues

(a) Specificity of the anti-CtBP1 antibody was evaluated in immunofluorescence assays using the CtBP1−/− vs. the CtBP1− rescued (CtBP1+) MEFs. Scale bar = 5 μm. (b) CtBP1 over-expression is an early event in melanoma. Melanoma cases with positive CtBP1 staining were reported as a percentage of the total cases for different pathological and clinical stages. (c) Human melanoma tissue array was stained for CtBP1. Vector Red Alkaline Phosphotase Substrate Kit 1 was used in the IHC development. Left panels show CtBP1-negative [CtBP1(−)] melanoma samples, while the right panels show CtBP1-positive [CtBP1(+)] melanoma samples. Melanin presence was observed as dark brown patchy staining (lower left panel). Scale bar = 40 μm.



CtBP1 Status	CtBP1 positive (n=43)	CtBP1 negative (n=13)
p16INK4a(+)	10	11
p16INK4a(-)	33	2

p=0.0001 between CtBP1 (+) and CtBP1 (-) groups, calculated by Fisher exact test.

Figure 2. CtBP1 represses *p16INK4a* expression in melanoma cells

(a) CtBP1 binding to the *p16INK4a* promoter. ** p<0.01 vs. IgG. (b) CtBP1 knockdown (siCtBP1-1 and siCtBP1-2) increased p16INK4a mRNA. (c) Nuclear p16INK4a staining was significantly increased by CtBP1 knockdown in A375 cells. Scale bar = 5 μm. (d) CtBP1 knockdown decreases proliferation of A375 cells. (e) Correlation between CtBP1 up-regulation and p16INK4a down-regulation in a human melanoma tissue array (US Biomax ME1003). Note that top panels show a lesion with negative CtBP1 but positive p16INK4a staining [p16INK4a(+)CtBP1(−)]. In contrast, a lesion with CtBP1 nuclear staining showed negative staining for p16INK4a in the consecutive section [lower panels, p16INK4a(−)CtBP1(+)]. Scale bar = 40 μm. p=0.0001 between CtBP1 (+) and CtBP1 (−) groups, calculated by Fisher's exact test.

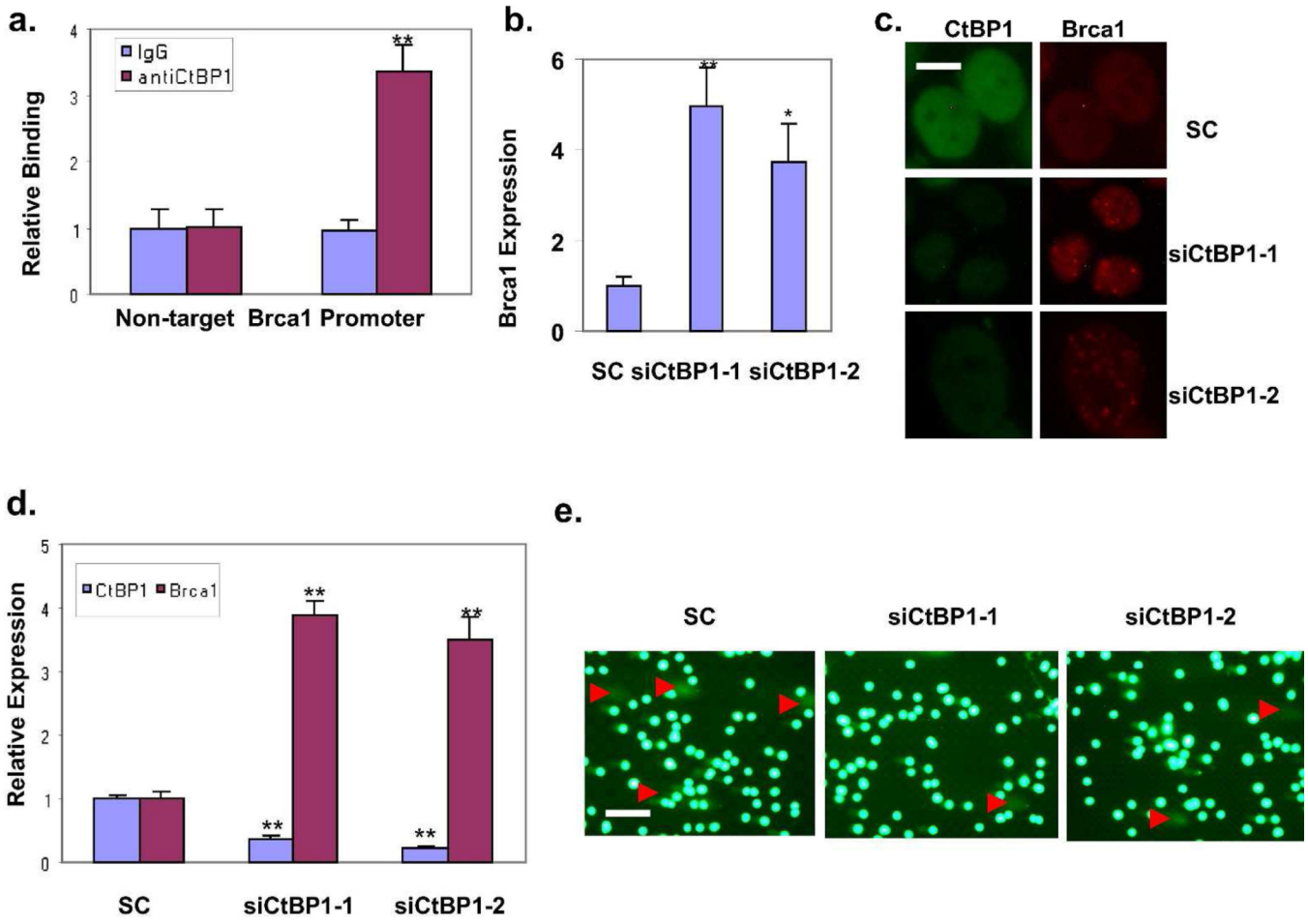


Figure 3. Transcriptional regulation of *Brca1* gene by CtBP1 in melanoma cells

(a) CtBP1 binding to the *Brca1* promoter in A375 cells. **p<0.01 vs. IgG. (b) CtBP1 knockdown (siCtBP1-1 and siCtBP1-2) in A375 cells increased Brca1 mRNA. (c) CtBP1 knockdown increases MMC-induced DNA repair foci formation. A375 cells were transfected with siRNAs for 48 hrs, and treated with 10 ng/ml MMC for 24 hrs before being subjected to DNA repair Brca1-foci staining. Scale bar = 5 μm. (d) Brca1 expression was up-regulated by *in vivo* siRNAs' delivery to knockdown CtBP1 in A375 xenografts for 2 weeks. (e) CtBP1 knockdown decreases DNA breaks. A375 cells were isolated from xenografts and assayed for DNA breaks. Arrows point to cells with accumulated DNA breaks (comets). Scale bar = 20 μm.

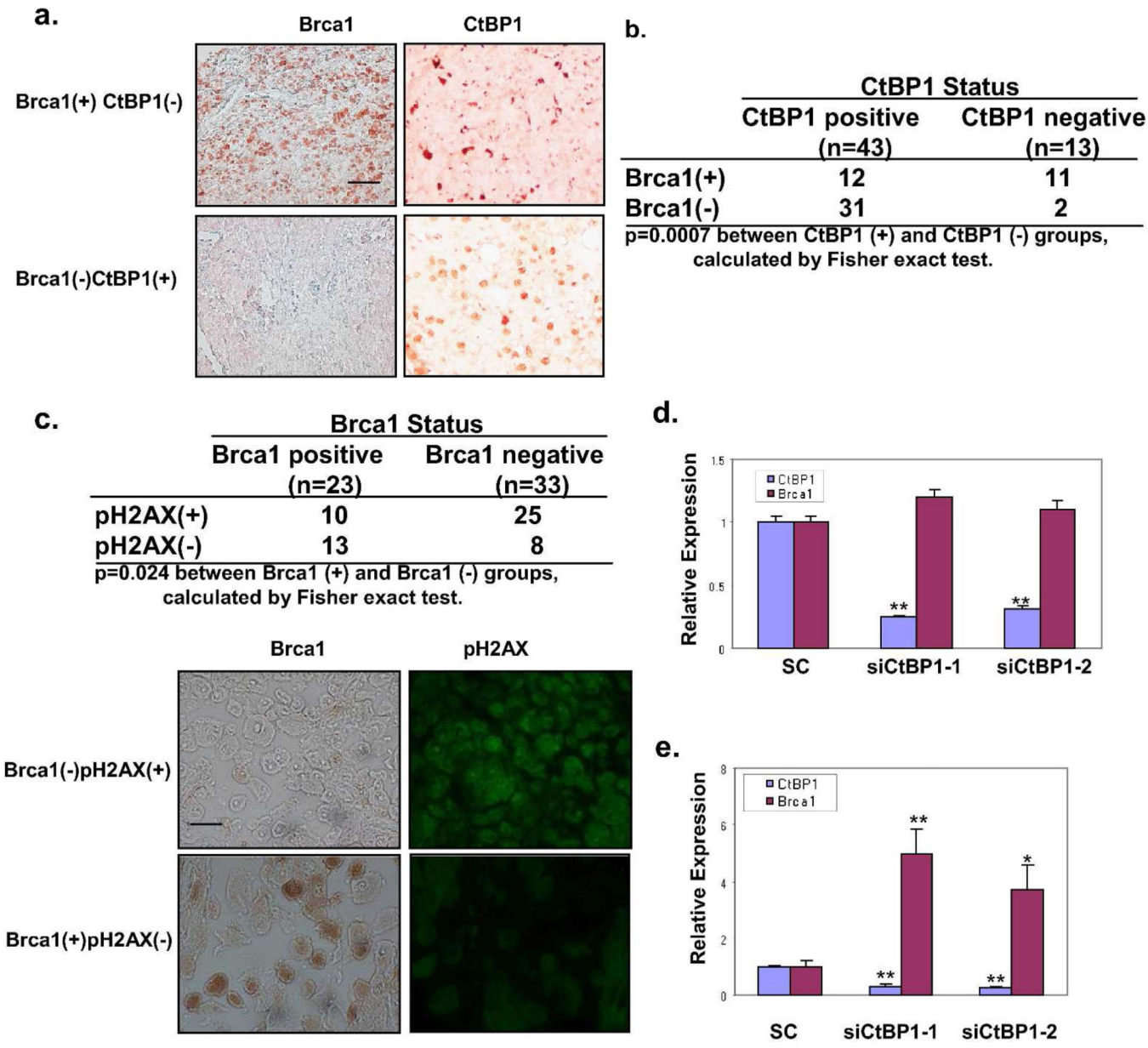


Figure 4. CtBP1 suppresses *Brca1* expression in melanoma samples

(a) Decreased Brca1 expression in CtBP1 over-expressed melanoma cases [lower panels, Brca1(−)CtBP1(+)] in a human melanoma tissue array (US Biomax ME1003). Scale bar = 40 μm. (b) Inverse correlation between CtBP1 and Brca1 expression in malignant melanoma lesions. p value between CtBP1 (+) and CtBP1 (−) groups was calculated by Fisher's exact test. (c) Correlation between pH2AX up-regulation and Brca1 down-regulation in malignant melanoma lesions. Scale bar = 10 μm. (d) Brca1 expression was not up-regulated by CtBP1 knockdown in MB1547 melanoma cells. (e) Brca1 expression was up-regulated by CtBP1 knockdown (siCtBP1-1 and siCtBP1-2) in MB1589 melanoma cells from patient sample engrafts.