ORIGINAL ARTICLE

Nucleus Accumbens-Associated 1 Contributes to Cortactin Deacetylation and Augments the Migration of Melanoma Cells

Kanako Tsunoda[1,2,3], Hiroki Oikawa[2], Hiroshi Tada[1], Yoshinori Tatemichi[1], Sosuke Muraoka[1,3], Shinpei Miura[1,3], Masahiko Shibazaki[1], Fumihiko Maeda[3], Kazuhiro Takahashi[3], Toshihide Akasaka[3], Tomoyuki Masuda[2] and Chihaya Maesawa[1]

We investigated the prognostic significance and post-transcriptional acetylation-modification of cortactin (CTTN) via the nucleus accumbens-associated 1 (NACC1)–histone deacetylase 6 (HDAC6) deacetylation system in primary melanomas and melanoma cell lines. Overexpression of CTTN protein was observed in 56 (73%) of 77 stage I–IV melanomas, and was significantly correlated with tumor thickness, lymph node metastasis, distant metastasis, and disease outcome. The patients whose tumors exhibited CTTN overexpression had a poorer outcome than patients without this feature ($P=0.028$, log-rank test). NACC1 and CTTN proteins, but not HDAC6, were overexpressed in four melanoma cell lines in comparison with a primary culture of normal human epidermal melanocytes. Knockdown of both *NACC1* and *HDAC6* markedly downregulated the migration activity of all melanoma cell lines ($P<0.05$), and induced a gain of CTTN protein acetylation status. Confocal microscopy showed that hyperacetylation of CTTN modulated by depletion of both *NACC1* and *HDAC6* induced disappearance of CTTN protein at the leading edge of migrating cells, resulting in stabilization of the focal adhesion structure and development of actin stress fibers. These data suggest that the acetylation status of CTTN modulated by the NACC1–HDAC6 deacetylation system induces acceleration of melanoma cell migration activity via an actin-dependent cellular process, possibly contributing to aggressive behavior (invasion/metastasis) of the melanoma cells.

Journal of Investigative Dermatology (2011) **131,** 1710–1719; doi:10.1038/jid.2011.110; published online 12 May 2011

INTRODUCTION

Cutaneous melanoma is one of the most aggressive solid tumors, and its incidence and mortality rate are currently increasing in most countries (Marks, 2000). The aggressiveness of malignant melanoma is characterized by high metastatic ability and resistance to chemotherapy and immunotherapy (Satyamoorthy and Herlyn, 2002; Soengas and Lowe, 2003; Postovit *et al.*, 2006; Gajewski, 2007). Tumor cell motility and migration are intrinsic components of tumor cell invasion and distant metastasis. Cell migration, motility, and adhesion, as well as tumor invasion and metastasis, are largely facilitated by remodeling of the actin cytoskeleton (van Rossum *et al.*, 2005).

Cortactin (official gene symbol, *CTTN*; and also as known *EMS1* and *FLJ34459*) is one of the most important protein molecules involved in actin-crosslinking (Rothschild *et al.*, 2006), and its overexpression has been postulated to mediate the increased invasive and metastatic activities of tumor cells because of its effects on the organization and function of the cytoskeleton and cell adhesion structures (Luo *et al.*, 2006). CTTN was originally identified as a protein substrate of Src tyrosine kinase, and has an important role in the regulation of cell motility (Wu and Parsons, 1993). It interacts with F-actin to promote polymerization and branching. CTTN can be found in areas of dynamic actin assembly, such as the leading edge of migrating cells (e.g., in lamellipodia and membrane ruffles; Wu and Parsons, 1993; Kaksonen *et al.*, 2000; Uruno *et al.*, 2001; Weaver *et al.*, 2001). Translocation of CTTN protein to the cell periphery requires activation of the small GTPase Rac1, and leads to activation of the actin-nucleating complex Arp2/3 (Weed *et al.*, 1998; Uruno *et al.*, 2001; Weaver *et al.*, 2001; Head *et al.*, 2003). Overexpression of CTTN increases cell migration activity (Patel *et al.*, 1998; Kowalski *et al.*, 2005), whereas depletion of *CTTN* impairs it (Bryce *et al.*, 2005).

[1]Division of Bioscience, Department of Tumor Biology, Center for Advanced Medical Science, Morioka, Japan; [2]Department of Pathology, School of Medicine, Iwate Medical University, Morioka, Japan and [3]Department of Dermatology, School of Medicine, Iwate Medical University, Morioka, Japan

Correspondence: Chihaya Maesawa, Division of Medical Bioscience, Department of Tumor Biology, Center for Advanced Medical Science, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan. E-mail: chihaya@iwate-med.ac.jp

Abbreviations: CTTN, *cortactin; F, Flag; FA, focal adhesion; IB, immunoblotting; IP, immunoprecipitation; M0, no distant metastasis; M1, metastasis to distant organs; N0, no metastases with lymph nodes; N1, one metastatic node; N2, two to three metastatic nodes; N3, ⩾ four metastatic nodes;* NACC1, *nucleus accumbens-associated 1; NHEM-M, normal human epidermal melanocyte; TSA, trichostatin A*

Received 17 December 2010; revised 4 February 2011; accepted 13 February 2011; published online 12 May 2011

© 2011 The Society for Investigative Dermatology

A (to our knowledge) previously unreported study has demonstrated that CTTN is the substrate for a unique member of the histone deacetylase family, HDAC6 (Zhang *et al.*, 2007), which is a cytoplasmic HDAC responsible for deacetylation of α-tubulin, the molecule present in cytoskeletal microbules. CTTN deacetylation by HDAC6 also enhances the ability of CTTN to bind with F-actin by modulating a ''charge patch'' in its repeat region, whereas acetylation of CTTN ablates the interaction between CTTN and F-actin, resulting in a decrease of cell migration.

On the other hand, nucleus accumbens-associated 1 (official gene symbol, *NACC1*; also as known *NAC1*; *BEND8, NAC-1*, *BTBD14B,* and *FLJ37383*) is a member of the BTB/POZ family and a transcriptional repressor associated with tumor cell growth, survival, and chemosensitivity. Nakayama *et al.* (2006) have demonstrated that *NACC1* is significantly overexpressed in several types of human carcinoma, and that intense NACC1 immunoreactivity is significantly correlated with tumor recurrence in ovarian cancer. They have also reported that NACC1 controls cell growth and the *GADD45-gip1* gene (Nakayama *et al.*, 2007). *NACC1* gene knockdown inhibits cell growth and induces apoptosis in uterine cervical cancer cell lines (Yeasmin *et al.*, 2008), and, moreover, NACC1 overexpression is well correlated with resistance to taxane derivatives (Ishibashi *et al.*, 2008), which are chemotherapeutic agents that act by binding to microtubules. NACC1 is not only an important prognostic biomarker in human cancers but also an attractive target for designing modulators of taxane sensitivity (Jinawath *et al.*, 2009).

Our group has recently found that NACC1 binds to HDAC6 directly and modulates the function of the latter. This interaction is necessary for microtubule deacetylation, with implications for tumor cell biology in the context of cytoskeleton-dependent cellular processes such as cell motility (unpublished data). We have hypothesized that the NACC1–HDAC6 system might also contribute to the deacetylation of CTTN at the leading edge.

In the present study, we show that CTTN overexpression is significantly associated with human melanoma progression and disease outcome. Also, we demonstrate biochemically that direct interaction between NACC1 and CTTN, and disruption of the NACC1–HDAC6 deacetylation system, affect the acetylation status of CTTN. Moreover, we demonstrate that the hyperacetylation status of CTTN induced by disruption of the NACC1–HDAC6 system prevents the translocation of CTTN protein to the cell periphery, blocks its association with F-actin, and impairs the motility of melanoma cells. In addition, it affects the turnover of the focal adhesion (FA). Our findings demonstrate a pathway by which actin-dependent cell motility can be modulated by the NACC1–HDAC6 deacetylation system in human melanoma cells.

RESULTS

Immunohistochemistry of CTTN protein

We immunohistochemically examined the expression of CTTN protein in 92 patients (including 15 patients at stage 0) with malignant melanoma. Immunoreactivity for CTTN protein was negative in epidermal melanocytes (Supplementary Figure S1a online), and varied among the primary malignant melanomas (Supplementary Figure S1b–d online). CTTN protein was expressed in the cytoplasm (Supplementary Figure S1d online). The tumors in the 15 patients at stage 0 all showed negative/faint. Positive immunoreactivity (medium, 21; strong, 35) was observed in 56 (73%) of the 77 melanomas at stage I–IV.

To evaluate whether CTTN expression in human primary melanomas correlates with patient outcome, we analyzed the relationship between CTTN protein expression and clinicopathological variables. We excluded 15 patients at stage 0.

Overexpression of CTTN was significantly correlated with tumor thickness, lymph node metastasis, distant metastasis, and disease outcome (Supplementary Table S1 online). We then carried out univariate analyses of clinicopathological variables (including CTTN expression) affecting the overall survival of patients (stage I–IV, Supplementary Table S2 online). There were significant relationships between some of the clinicopathological variables examined (tumor thickness, lymph node status, distant metastasis, tumor stage, and CTTN expression) and patient outcome (Supplementary Table S2 online).

After a median follow-up of 40 months (range, 2–147 months), 26 patients (34%) had died because of disease relapse. Kaplan–Meier curves showed a trend toward worse outcome in patients whose tumors showed CTTN immunoreactivity compared with patients whose tumors lacked it ($P=0.028$, log-rank test, Figure 1). We then examined whether CTTN expression was an independent prognostic factor in patients with melanomas. We performed multivariate analysis focusing on the presence of ulceration, tumor thickness, lymph node metastasis, distant metastasis, and expression of CTTN. Lymph node metastasis was the only variable that was independently associated with patient survival; immunoreactivity for CTTN showed no such association (Supplementary Table S3 online). This indicated

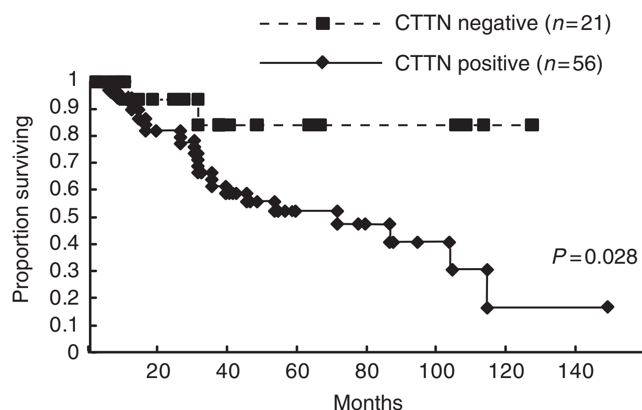


Figure 1. Survival curves of 77 melanoma patients according to cortactin (CTTN) immunoreactivity. Kaplan–Meier estimates of survival among 77 patients with malignant melanomas with ($n=56$) and without ($n=21$) CTTN immunoreactivity (stage I–IV). Outcome shows a tendency to be worse for patients whose tumors had CTTN immunoreactivity than for those whose tumors lacked it ($P=0.028$, log-rank test).

that CTTN overexpression is not an independent indicator of overall patient survival.

Expression of CTTN, NACC1, and HDAC6 in melanoma cell lines

We investigated the expression of CTTN, NACC1, and HDAC6 protein in four melanoma cell lines (HMV II, SK-MEL-28, CRL1579, and G-361) and primary-cultured normal human epidermal melanocytes (NHEM-M; Supplementary Figure S2a online). The four malignant melanoma cell lines strongly expressed NACC1 and CTTN proteins, whereas the expression of these proteins in NHEM-M was weak (Supplementary Figure S2b and d online). The expression of HDAC6 protein showed no differences between the malignant melanoma cell lines and epidermal melanocytes (Supplementary Figure S2c online). Expression of CTTN and NACC1 protein was increased in malignant melanoma cells in comparison with NHEM-M.

Migration activity induced by treatment with *NACC1*-small interfering RNAs in melanoma cell lines

We first evaluated the knockdown efficiency of *NACC1*-small interfering RNAs (siRNAs; #1, #2, and #3; 10 nM) in a malignant melanoma cell line (CRL1579). In comparison with negative control-siRNA, all the siRNAs caused 75–90% downregulation of *NACC1* mRNA expression (Figure 2a). NACC1 protein expression was analyzed by western blotting at 48 hours after siRNA treatment. All the siRNAs caused 60–80% downregulation of NACC1 protein expression. One siRNA (#1) was able to downregulate NACC1 protein by 80% in comparison with the negative control (Figure 2b). We then examined phenotypic changes in the migration activities of the four melanoma cell lines (HMV II, SK-MEL-28, CRL1579, and G-361) using #1 *NACC1*-siRNA. *NACC1* knockdown significantly decreased melanoma cell migration activities in comparison with the negative control at 24 and 48 hours ($P<0.05$, Mann–Whitney *U*-test; Figure 2d). Expression of

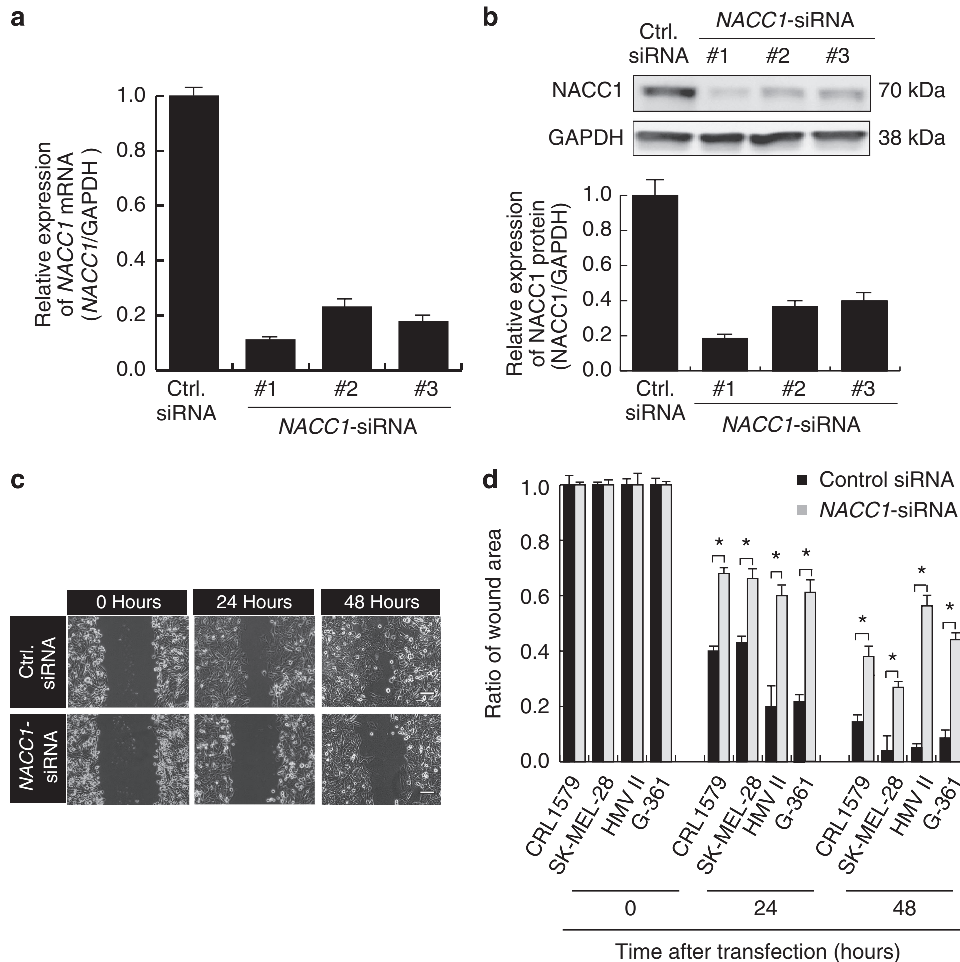


Figure 2. *Nucleus accumbens-associated 1* (*NACC1*) knockdown and its effects on migration activity of melanoma cell lines. (**a**) *NACC1* mRNA expression was evaluated by real-time quantitative PCR. A decrease of relative *NACC1* mRNA expression of over 75% was observed at 48 hours after transfection of all three *NACC1*-siRNAs (10 nM; information on siRNA sequences #1, #2, and #3 is available on the ABI website) in comparison with control (Ctrl.) siRNA. (**b**) Immunoblotting for NACC1 protein (top) and its quantification (bottom) at 48 hours after siRNA transfection. (**c**) Photographs of scratch assays for CRL1579 (bars = 100 μm) and (**d**) quantification four melanoma cell lines at 24 and 48 hours after transfection with *NACC1*- or control-siRNAs (10 nM). Cell migration activity (inversely correlated with the wound area ratio) was significantly decreased in cells treated with *NACC1*-siRNA at 24 and 48 hours. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA.

HDAC6 and CTTN was not altered by the *NACC1*-siRNA treatment (Supplementary Figure S3 online).

Furthermore, we also treated melanoma cell lines with siRNAs for *HDAC6* and *CTTN*. Both siRNAs significantly decreased cell migration activities (Supplementary Figure S4 online).

Modulation of CTTN acetylation status by NACC1

We examined whether endogenous CTTN protein was acetylated by *NACC1*-siRNA knockdown using immunoprecipitation (IP) and subsequent western blotting. Experiments were performed to examine the acetylation status of CTTN protein according to those for *HDAC6*-siRNA, as described previously (Zhang *et al.*, 2007). First, we treated CRL1579 cells with the class I and II HDAC inhibitor, trichostatin A (TSA), followed by IP with anti-acetylated-lysine antibody, and probing with anti-CTTN antibody (Figure 3a). This clearly demonstrated an increase in the acetylation status of CTTN protein (Figure 3a). On other hand, the hyperacetylation status of cortatin resulting from the TSA treatment was also confirmed by IP with anti-CTTN antibody followed by anti-acetyl-lysine antibody (Figure 3a, right). Similar results were obtained in CRL1579 cells with *HDAC6*-siRNA treatment, as described previously (Figure 3b; Zhang *et al.*, 2007).

We then examined the effects of *NACC1*-siRNA treatment in CRL1579 cells. Endogenous CTTN protein was found to be hyperacetylated by IP of a whole cell extract with *NACC1*-knockdown either with an anti-acetylated-lysine antibody followed by western blotting with an anti-CTTN antibody (Figure 3c) or anti-CTTN followed by anti-acetyl-lysine antibody (Figure 3c).

In vitro and *in vivo* interactions between NACC1 and CTTN proteins

We then examined the physical association between NACC1 and CTTN using pulldown assays. We synthesized the full-length Flag-NACC1 (prey) and Halo-CTTN (bait) proteins using the wheat germ protein system. As shown in Supplementary Figure S5 online, full-length CTTN interacted with full-length NACC1 *in vitro*.

Next, we examined the *in vivo* interaction between CTTN and NACC1, and determined the interaction domains by using IP and subsequent western blotting. A schematic representation of the *NACC1* construct is shown in Figure 4a. The *NACC1* gene has a BTB/POZ domain at the N-terminus, and a recently assigned domain, BEN, at the C-terminus (Abhiman *et al.*, 2008). The biological significance of the BEN domain remains unclear. A previous report showed that either the BTB/POZ or the non-BTB/POZ domain was able to bind to HDAC3 and HDAC4 (Korutla *et al.*, 2005). Constructs of *CTTN* are shown in Figure 4b. *CTTN* contains an N-terminal acidic domain, six and a half tandem repeats of a unique 37-amino-acid sequence (repeat), and a *Src* homology 3 domain at the C-terminal (Wu and Parsons, 1993). The repeat region of CTTN is both necessary and sufficient for F-actin binding.

In vivo interaction between full-length NACC1 and CTTN was confirmed by IP–western blot (lane 1 in each Figure 4c and d), similarly to the *in vitro* data (Supplementary Figure S5 online). To determine the NACC1 domain that binds CTTN, we carried out co-transfection of deleted NACC1 and full-length CTTN constructs in CRL1579. Full-length Flag-CTTN interacted with only the BTB/POZ domain (NACC1/1–133) and the N-terminal two-thirds of NACC1 (NACC1/1–360

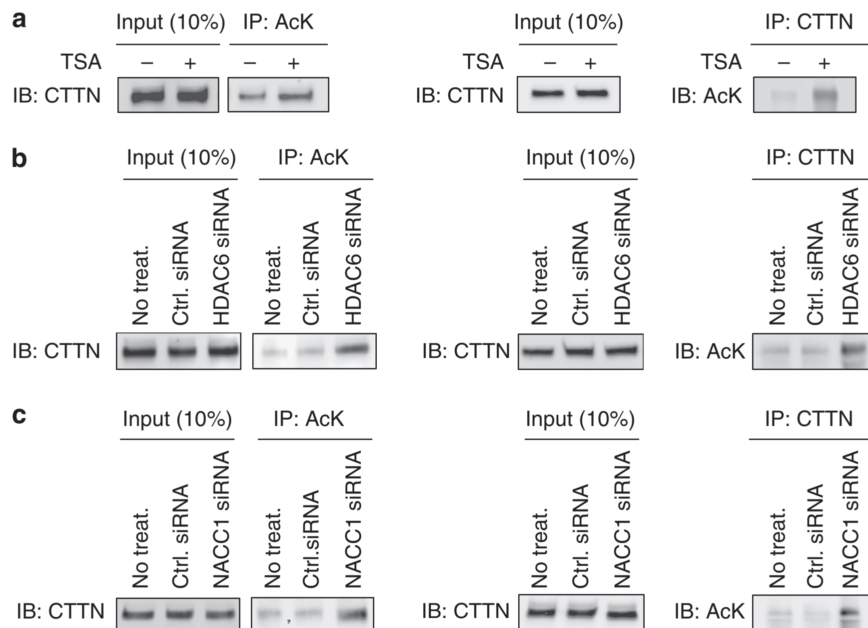


Figure 3. Acetylation status of endogenous cortactin (CTTN) protein evaluated by immunoprecipitation followed by western blotting. Gain of CTTN acetylation modification was observed in CRL1597 cells after treatment with a histone deacetylase inhibitor, trichostatin A (TSA) (**a**), and small interfering RNAs (siRNAs) for *histone deacetylase 6* (*HDAC6*)- (**b**) and *nucleus accumbens-associated 1* (*NACC1*)- (**c**). All IP–western experiments were performed 48 hours after transfection with TSA or siRNA (10 nM). AcK, anti-acetyl-lysine antibody; IB, immunoblotting; IP, immunoprecipitation; No treat., no treatment.

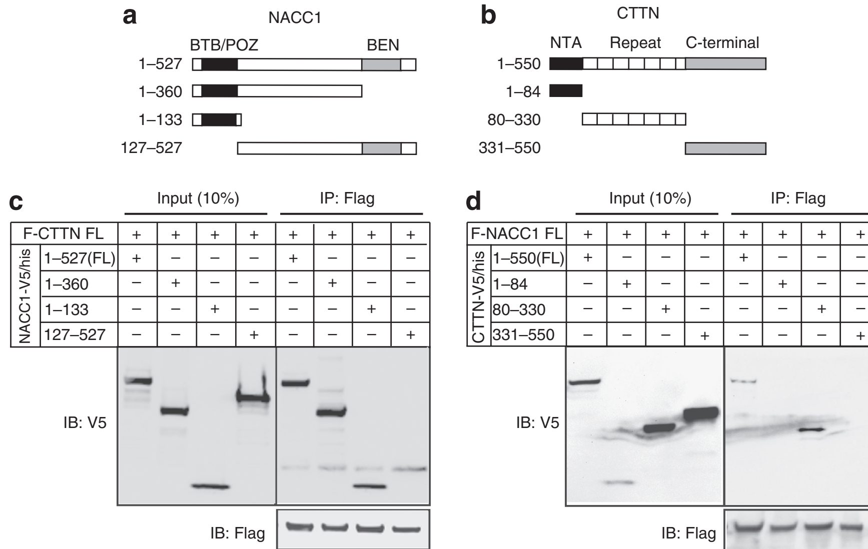


Figure 4. Co-immunoprecipitation assays for detection of nucleus accumbens-associated 1 (NACC1) binding to cortactin (CTTN). Co-transfection with epitope-tagged (Flag-NACC1 and CTTN-V5/His) constructs was performed, followed by immunoprecipitation (IP) and immunoblotting (IB). (**a**) Schematic representation of full-length NACC1 and its deletion mutants: BTB/POZ, bric-a-brac tramtrack broad complex/pox virus and Zn finger; BEN, BANP, E5R, and NAC1, whose function is unclear. (**b**) Schematic representation of full-length CTTN and deletion mutants. NTA, N-terminal acidic domain; repeat, C-terminal. The BTB/POZ domain interacts with full-length (FL) CTTN (**c**), and the repeat region of CTTN binds to FL NACC1 (**d**).

including the BTB/POZ domain; Figure 4c). The C-terminal two-thirds of NACC1 (NACC1/127–527) was unable to bind to full-length CTTN. Our data thus confirmed that CTTN interacts specifically with the BTB/POZ domain of NACC1.

We next examined the regions of CTTN binding to full-length NACC1. Full-length Flag-NACC1 and full-length CTTN-V5/His, the N-terminal acidic domain (CTTN/1–84), and the repeat (CTTN/80–330) or the C-terminal (CTTN/331–550) were co-transfected into CRL1579 cells. Full-length Flag-NACC1 interacted with the repeat region of CTTN (Figure 4d). The N-terminal acidic domain and C-terminal of CTTN were unable to bind to full-length NACC1. Our data thus showed that NACC1 interacts specifically with the repeat region of CTTN.

Change in subcellular localization of CTTN on *NACC1* knockdown

CTTN interacts with F-actin to promote polymerization and branching. CTTN can be found in areas of dynamic actin assembly, such as the leading edge of cell migration (e.g., in lamellipodia and membrane ruffles; Wu and Parsons, 1993; Kaksonen *et al.*, 2000; Uruno *et al.*, 2001; Weaver *et al.*, 2001). Using immunofluorescence, we investigated whether knockdown of *NACC1* affects its subcellular location (Figure 5). We first examined the localization of CTTN and actin filaments treated with control-siRNA (Figure 5). In melanoma cells, CTTN became localized at the cell periphery and was colocalized with phalloidin-stained actin filaments (Figure 5). Treatment with *CTTN*-siRNA induced the development of actin stress fibers (Figure 5). Next, we examined the localization of CTTN and actin filaments after treatment with *HDAC6*- or *NACC1*-siRNA. Diffuse punctate CTTN staining and development of actin stress fibers were detected (Figure 5). A previous study has described that acetylated CTTN is unable to effectively interact with CTTN (Zhang *et al.*, 2007). Our results showed that knockdown of *HDAC6* and *NACC1* increased the acetylation level of CTTN. The acetylation of CTTN resulting from disruption of the NACC1–HDAC6 deacetylation system decreased actin-binding activity at the cell periphery. Disappearance of CTTN was evident at the cell periphery, but the protein was visualized in the cytoplasm (Figure 5).

Involvement of focal adhesion formation resulting from *NACC1* knockdown

We were interested in FA formation in cells treated with *NACC1*-siRNA. First, we examined the expression of CTTN, HDAC6, and vinculin proteins at 48 hours after *NACC1*-siRNA treatment (Supplementary Figure S3 online). No marked changes in the expression of any of the proteins were observed under *NACC1* knockdown. Thus, *NACC1* knockdown did not appear to affect the transcriptional regulation of CTTN, HDAC6, and vinculin.

Next, we investigated whether *NACC1* and *HDAC6* knockdown affected FA formation and actin fibers. FA was visualized by vinculin immunostaining (Figure 6a). We first examined the status of FA and actin fibers under treatment with *CTTN*-siRNA in CRL1579. The area and the number of FA were significantly increased in CTTN knockdown cells in comparison with cells treated with control-siRNA (Figure 6a). Actin stress fibers were observed in CTTN knockdown cells (Figure 6a). Treatment with an HDAC inhibitor, TSA, also increased the number (Supplementary Figure S6 online and Figure 6b) and the area (Supplementary Figure S6 online and

Figure 6c) of FA in CRL1579 cells. Actin stress fibers were observed in CRL1579 cells treated with TSA, as well as in those treated with *CTTN*-siRNA (Supplementary Figure S6 online).

Furthermore, we examined the status of FA and actin dynamicity after treatment with *HDAC6*- and *NACC1*-siRNA. *NACC1*- and *HDAC6*-knockdown cells also showed a significant increase in the number (Figure 6b) and area (Figure 6c) of FA. Thus, NACC1 appears to regulate actin-dependent cell motility through modulation of the acetylation status of CTTN, and moreover, promotes the formation of FA.

DISCUSSION

Accumulated evidence supports the notion that CTTN is involved in tumor metastasis (Buday and Downward, 2007). The human homolog of CTTN is amplified at chromosome 11q13 in several types of tumors (Schuuring *et al.*, 1993; Bringuier *et al.*, 1996; Patel *et al.*, 1996; Yuan *et al.*, 2003). Rodrigo *et al.* (2000) identified *CTTN* amplification in 20% (21/104) of patients with head and neck squamous cell carcinomas, and found that these tumors exhibited aggressive behavior such as an advanced T stage, lymph node metastasis, poor histological differentiation, and recurrence.

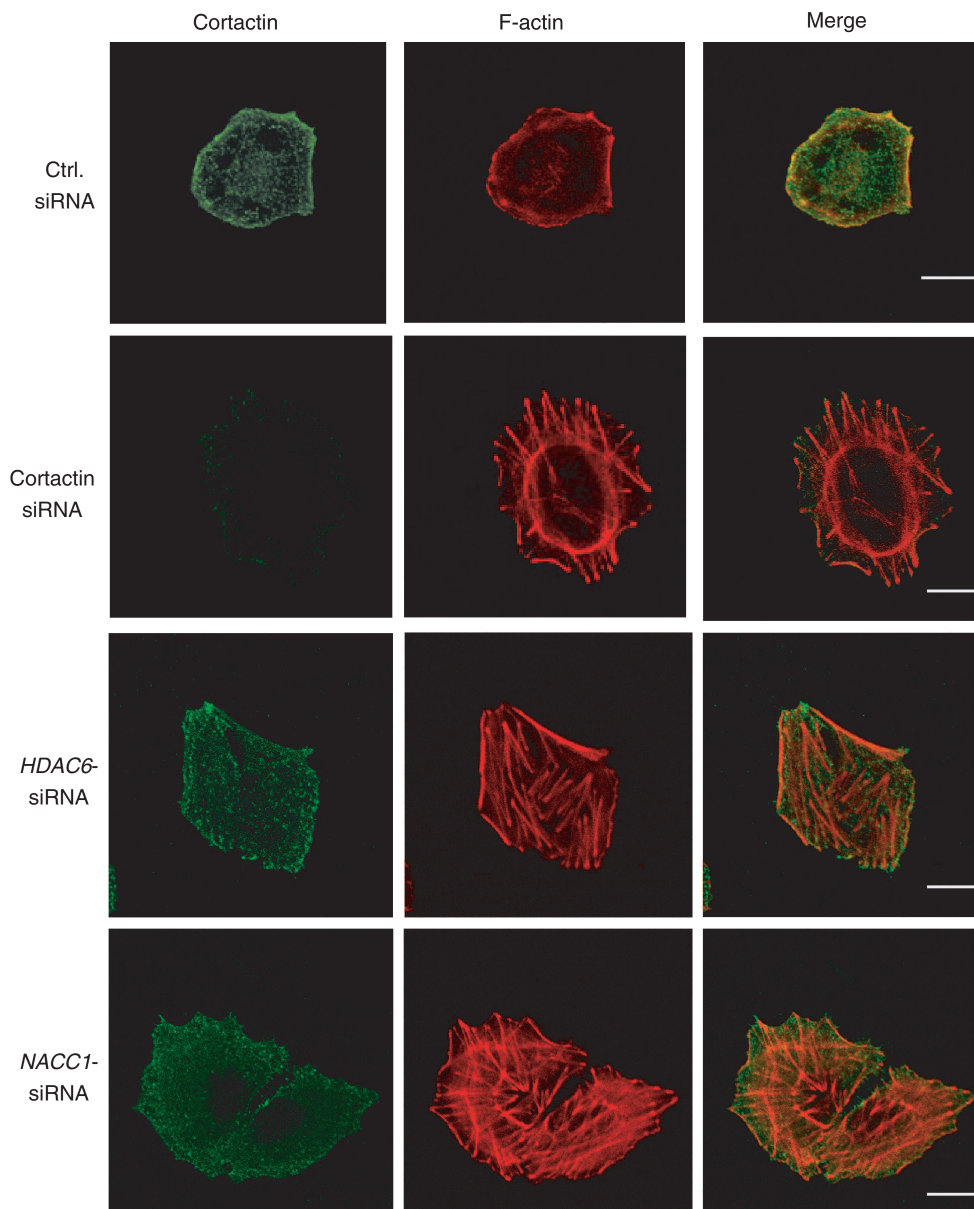
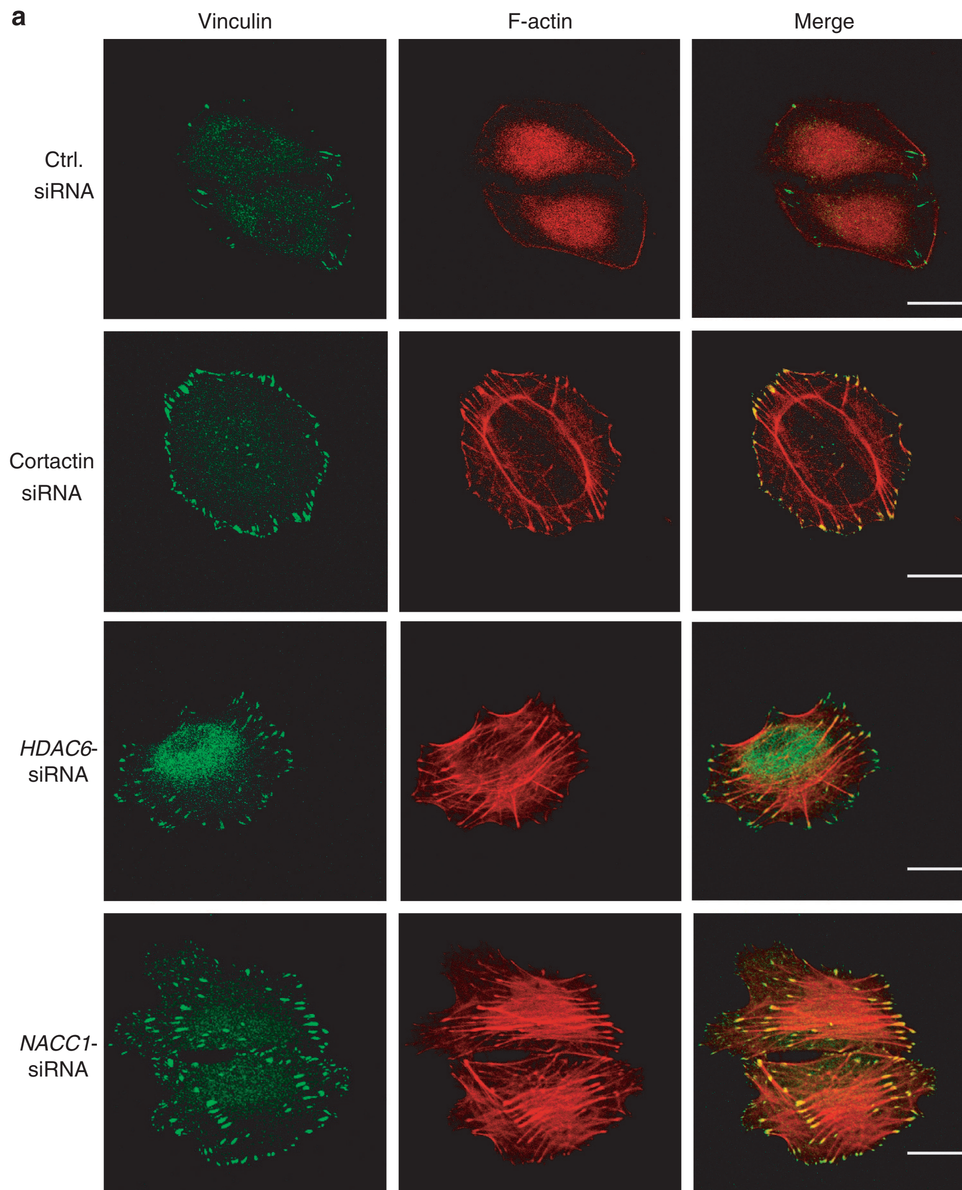
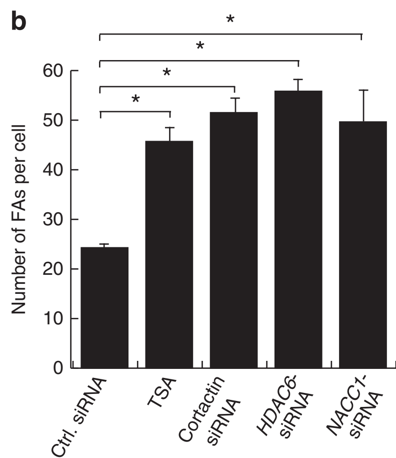
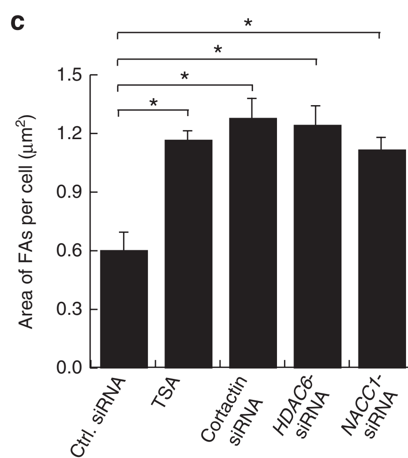


Figure 5. Confocal microscopy observations of cortactin (CTTN, green) and F-actin (red) in CRL1579 cells treated with small interfering RNAs (siRNAs) for *CTTN, histone deacetylase 6 (HDAC6)*, and *nucleus accumbens-associated 1* (*NACC1*). Both CTTN protein and F-actin were observed at the cell periphery, and well merged in CRL1579 cells treated with control (Ctrl.) siRNA (48 hours after siRNA treatment). In these cells, no stress fiber development was visualized. *CTTN*-siRNA knockdown induced the development of stress fibers, as was the case with *HDAC6*- and *NACC1*-siRNA treatments. Disappearance of cortacin protein at the leading edge was observed in cells treated with *NACC1*- and *HDAC6*-siRNA. In these cells, development of actin stress fibers was observed, as was the case with *CTTN*-siRNA treatment. Bars = 20 μm.


a
Vinculin
F-actin
Merge
Ctrl.
siRNA
Cortactin
siRNA
HDAC6-
siRNA
NACC1-
siRNA


b
Number of FAs per cell
0
10
20
30
40
50
60
*
Ctrl. siRNA
TSA
Cortactin siRNA
HDAC6-siRNA
NACC1-siRNA


c
Area of FAs per cell (μm²)
0.0
0.3
0.6
0.9
1.2
1.5
*
Ctrl. siRNA
TSA
Cortactin siRNA
HDAC6-siRNA
NACC1-siRNA

Although coamplification of *CTTN* and *cyclin D1* (official gene symbol, *CCND1*) was observed, *CTTN* amplification was an independent predictor of disease outcome. Furthermore, overexpression of CTTN is correlated with lymph node metastasis and poor prognosis (Gibcus *et al.*, 2008; Wang *et al.*, 2009, 2010; Yamada *et al.*, 2010). In cultured fibroblasts, CTTN is distributed mainly along the leading edges of cells, e.g., in lamellipodia and punctate-like structures (Bowden *et al.*, 1999). Overexpression of CTTN in NIH3T3 cells leads to enhanced cell motility and invasion (Patel *et al.*, 1998). In MDA-MB-231, an invasive breast cancer cell line, CTTN is associated with the invadopodium, a cortical structure that penetrates into the extracellular matrix during invasion, and overexpression of CTTN in this cell line promotes cell migration (Bowden *et al.*, 1999). Moreover, CTTN potentiates bone metastasis of breast cancer cells in nude mice (Li *et al.*, 2001).

Our data also provided evidence that post-transcriptional modification of CTTN *via* the NACC1–HDAC6 deacetylation system has an important role in melanoma cell migration. Zhang *et al.* (2007) have demonstrated that CTTN is acetylated *in vivo* and is a genuine substrate of HDAC6, which interacts directly with the repeat region of CTTN through its two catalytic domains. CTTN deacetylation enhances the ability of CTTN to bind F-actin by modulating a ''charge patch'' in its repeat region, whereas acetylation of CTTN ablates the interaction between CTTN and F-actin, resulting in decreased cell migration. Using IP followed by western blotting, *in vitro* pulldown assay and use of a yeast two-hybrid system, we have found that NACC1 protein binds directly to HDAC6 (unpublished data). Also, using a cell-free system, we have also confirmed that NACC1 protein itself has no deacetylase activity *in vitro*. In fact, the amino-acid sequence of NACC1 includes no deacetylase catalytic domain. However, the NACC1 protein complex pulled down by epitope-tagged *NACC1* from cultured cells has been shown to exhibit deacetylation activity on porcine brain acetylated tubulin (unpublished data). The microtubule-acetylation is a substrate for the NACC1–HDAC6 interaction system, indicating that the NACC1–HDAC deacetylation system is also involved in modification of CTTN protein acetylation. NACC1 might also contribute to deacetylation of CTTN at the leading edge, and both microtubule- and actin-dependent processes involved in cell motility might be modulated by this deacetylation system. A previous study has demonstrated that CTTN is also the substrate of SIRT1 (Zhang *et al.*, 2009). In a future study, we will need to examine the association between SIRT1 and NACC1.

NACC1 has recently been highlighted not only as a key player in neurogenesis and cancer biology (Nakayama *et al.*, 2006, 2007; Shen *et al.*, 2007), but also as a transcription factor for the maintenance of pluripotency of ES and other types of stem cells (Kim *et al.*, 2008). NACC1 binds with HDAC3 and HDAC4 (Korutla *et al.*, 2005), and acts mainly as a transcriptional repressor. HDAC3 belongs to the class I HDACs, being localized mainly within the nucleus, and forms complexes with other transcriptional repressor proteins such as MeCP2 (Kim *et al.*, 2007). The class II HDACs can be further subdivided into class IIa (HDAC4, 5, 7, and 9) and IIb HDACs (HDAC6 and 10). Similar to class I HDACs, class IIa HDACs act as transcriptional repressors, mainly in the nucleus (Yang and Gregoire, 2005). Therefore, they may lack the deacetylation activity on CTTN protein. In fact, Zhang *et al.* (2007) have already demonstrated that HDAC6 modulates cell motility by altering the acetylation level of CTTN, and that no other ectopically expressed class II HDACs have any effect on CTTN acetylation. The functions of the BTB/POZ family have been investigated almost entirely in the context of gene expression and chromatin dynamics, whereas our present study has provided a new perspective on the function of *NACC1*, indicating that it can regulate actin-related protein-dependent cellular processes by its interaction with HDAC6 protein.

CTTN-knockdown cells have a selective defect in the rate of formation of new adhesions in lamellipodial protraction (Bryce *et al.*, 2005). Several proteins are found at both the lamellipodium and in FA. Adhesion regulates Mena/VASP phosphorylation (Howe *et al.*, 2002), which affects the ability of Mena/VASP proteins to bind to actin, *Src* homology 3 domains (Lambrechts *et al.*, 2000), and the tyrosine kinase, Abl. (Howe *et al.*, 2002). CTTN is localized at lamellipodia and FA, and CTTN phosphorylation regulates FA dynamics and lamellipodial protrusion. The action of tyrosine-phosphorylated CTTN on FA stability and turnover could have profound effects on actin stress fiber dynamics, and thereby markedly retard or accelerate cell migration (Kruchten *et al.*, 2008). Our results indicate that hyper-acetylation of CTTN protein modulated by the NACC1–HDAC6 system has a dramatic effect on the development of actin stress fibers and FA formation. We found that the size and number of FA in cells were increased when CTTN was hyperacetylated because of disruption of NACC1–HDAC6 deacetylation activity as well as TSA treatment. As well as yielding hyperacetylated CTTN, treatment with TSA induced an equivalent increase in cellular adhesions. These data suggest that hyperacetylation of CTTN appears to be both necessary and sufficient for increasing the degree of cellular adhesion, possibly participating in control of the FA turnover rate. The post-transcriptional modification (acetylation and phosphorylation) of NACC1 and other proteins (FA kinase, paxillin, talin, etc.) and its association with FA turnover is an issue that clearly warrants further study.

A, to our knowledge, previously unreported study has shown that, in malignant melanoma with metastatic spread, a

Figure 6. Confocal microscopy observations of focal adhesion (FA, vinculin, green) and F-actin (red), and quantification of the number and area of FA, in CRL1579 cells treated with small interfering RNAs (siRNAs) for *CTTN, histone deacetylase 6 (HDAC6),* and *nucleus accumbens-associated 1* (*NACC1*). An increase of FA formation, in terms of both number (**a**, **b**) and area (**a**, **c**) visualized by vinculin immunostaining (**a**), was observed. One hundred cells were examined for each treatment. Along with the increase of FA formation, development of actin stress fibers was observed. Bars = 20 μm.

high level of CTTN expression is correlated with poor outcome (Xu *et al.*, 2010). Indeed, in our present study, CTTN overexpression was also associated with tumor thickness, lymph node metastasis, and distant metastasis. Furthermore, patients with CTTN-positive tumors had worse survival than patients whose tumors showed negative/weak CTTN expression. Thus, CTTN overexpression has significance as a prognostic biomarker in patients with malignant melanomas. On the basis of our present results and previous studies (Zhang *et al.*, 2007, 2009), we think that it will be necessary to evaluate the acetylation status of CTTN in samples from patients. The next issue will be to clarify whether less invasive (N0, M0) tumors have lower CTTN expression than invasive (N1–3, M1) ones. For this purpose, it will be necessary to generate a monoclonal antibody specific to acetylated CTTN, as currently no such specific antibody is commercially available. We are currently attempting to determine the deacetylation lysine(s) of CTTN using mass spectrometry, and hope to generate an antibody specific to acetylated CTTN in the near future.

In relation to the management of patients with malignant melanoma, our present results provide two important insights into modification of CTTN by the NACC1–HDAC6 deacetylation system: (i) CTTN expression appears to be significantly correlated with lymph node metastasis and prognosis, whereas the only independent indicator of patient survival is lymph node metastasis, and (ii) CTTN hyperacetylation modulated by the NACC1–HDAC6 deacetylation system enhances the migration activity of melanoma cells, and may be a possible target for treatment of melanoma patients.

MATERIALS AND METHODS

Antibodies, reagents, and plasmids

Trichostatin A (Sigma Chemical, Saint Louis, MO) was prepared at a stock concentration of 6.6 mM in ethanol. For immunohistochemistry of surgical specimens, mouse monoclonal clone 30/cortactin (diluted 1:100; BD Transduction Laboratories, San Diego, CA) was used. For immunofluorescence, monoclonal anti-cortactin antibody clone 4F11 (diluted 1:200; Millipore Corporation, Bedford, MA) and monoclonal anti-vinculin clone hVIN-1 (1:400; Sigma Chemical) were used. The secondary antibody was Alexa Fluor 488 anti-mouse IgG (1:250; Invitrogen, Carlsbad, CA). For IP and/or immunoblotting (IB) of CTTN and vinculin, we used the same primary antibody as that used for immunofluoresence (CTTN; IP, 20 μg; IB, 1:1,000, vinculin; IB, 1:1,000). The other primary antibodies used were polyclonal NACC1 antibody (IP, 20 μg; IB, 1:300; Abcam, Cambridge, UK), polyclonal HDAC6 (H300) antibody (IB, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal acetylated-lysine antibody (IP, 20 μg; Cell Signaling Technology, Danvers, MA), monoclonal acetylated-lysine antibody clone 4G12 (IB, 1:1,000; Millipore Corporation), monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase, Clone 1D4 (IB, 1:1,000; Covance, Princeton, NJ), monoclonal Flag M2 antibody (IP, 20 μg; IB, 1:5,000; Sigma Chemical), monoclonal V5 antibody (IB, 1:5,000; Invitrogen), monoclonal His antibody (IP, 5 μg; Invitrogen) and polyclonal Anti-HaloTag antibody (IB, 1:1,000; Promega, Madison, WI). Complementary DNAs encoding full-length and partial sequences of NACC1 or CTTN were amplified by PCR, and inserted into mammalian expression vectors. Plasmids expressing FLAG (F)-NACC1 and CTTN, NACC1 and CTTN-V5-His (V5/His) were made using p3XFLAG-CMV-10 (Sigma Chemical), and pcDNA-DEST40 (Invitrogen), respectively. For the synthesis of recombinant proteins by the wheat germ extract system, pF3K WG (BYDV) Flexi Vector and pFN19A (HaloTag 7) T7 SP6 Flexi Vector (Promega) were used for F-NACC1 and Halo (H)-CTTN proteins, respectively. For immunofluorescence, Alexa Fluor 594 phalloidin (Invitrogen) and 4′-6-diamino-2-phenylindole (diluted 1:1,000; Wako, Osaka, Japan) solution were used.

Surgical specimens of malignant melanoma, immunohistochemistry, RNA isolation, reverse transcriptase quantitative PCR, siRNA knockdown, cell culture, transfection, IP, western blotting, *in vitro* transcription–translation, pulldown assay, tumor cell migration assay, confocal microscopy, and statistical analysis methods are described in Supplementary Materials online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research (30509013, 10326647, 10326647, and 16791346), and the MIAST (Medical Innovation by Advanced Science and Technology; 2010–2014) project, Grant-in-Aid for Strategic Medical Science Research Center from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Abhiman S, Iyer LM, Aravind L (2008) BEN: a novel domain in chromatin factors and DNA viral proteins. *Bioinformatics* 24:458–61

Bowden ET, Barth M, Thomas D *et al.* (1999) An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation. *Oncogene* 18:4440–9

Bringuier PP, Tamimi Y, Schuuring E *et al.* (1996) Expression of cyclin D1 and EMS1 in bladder tumours; relationship with chromosome 11q13 amplification. *Oncogene* 12:1747–53

Bryce NS, Clark ES, Leysath JL *et al.* (2005) Cortactin promotes cell motility by enhancing lamellipodial persistence. *Curr Biol* 15:1276–85

Buday L, Downward J (2007) Roles of cortactin in tumor pathogenesis. *Biochim Biophys Acta* 1775:263–73

Gajewski TF (2007) Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. *Clin Cancer Res* 13:5256–61

Gibcus JH, Mastik MF, Menkema L *et al.* (2008) Cortactin expression predicts poor survival in laryngeal carcinoma. *Br J Cancer* 98:950–5

Head JA, Jiang D, Li M *et al.* (2003) Cortactin tyrosine phosphorylation requires Rac1 activity and association with the cortical actin cytoskeleton. *Mol Biol Cell* 14:3216–29

Howe AK, Hogan BP, Juliano RL (2002) Regulation of vasodilator-stimulated phosphoprotein phosphorylation and interaction with Abl by protein kinase A and cell adhesion. *J Biol Chem* 277:38121–6

Ishibashi M, Nakayama K, Yeasmin S *et al.* (2008) A BTB/POZ gene, NAC-1, a tumor recurrence-associated gene, as a potential target for Taxol resistance in ovarian cancer. *Clin Cancer Res* 14:3149–55

Jinawath N, Vasoontara C, Yap KL *et al.* (2009) NAC-1, a potential stem cell pluripotency factor, contributes to paclitaxel resistance in ovarian cancer through inactivating Gadd45 pathway. *Oncogene* 28:1941–8

Kaksonen M, Peng HB, Rauvala H (2000) Association of cortactin with dynamic actin in lamellipodia and on endosomal vesicles. *J Cell Sci* 113(Pt 24):4421–6

Kim J, Chu J, Shen X *et al.* (2008) An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* 132:1049–61

Kim YB, Lee SY, Ye SK *et al.* (2007) Epigenetic regulation of integrin-linked kinase expression depending on adhesion of gastric carcinoma cells. *Am J Physiol Cell Physiol* 292:C857–66

Korutla L, Wang PJ, Mackler SA (2005) The POZ/BTB protein NAC1 interacts with two different histone deacetylases in neuronal-like cultures. *J Neurochem* 94:786–93

Kowalski JR, Egile C, Gil S *et al.* (2005) Cortactin regulates cell migration through activation of N-WASP. *J Cell Sci* 118:79–87

Kruchten AE, Krueger EW, Wang Y *et al.* (2008) Distinct phospho-forms of cortactin differentially regulate actin polymerization and focal adhesions. *Am J Physiol Cell Physiol* 295:C1113–22

Lambrechts A, Kwiatkowski AV, Lanier LM *et al.* (2000) cAMP-dependent protein kinase phosphorylation of EVL, a Mena/VASP relative, regulates its interaction with actin and SH3 domains. *J Biol Chem* 275:36143–51

Li Y, Tondravi M, Liu J *et al.* (2001) Cortactin potentiates bone metastasis of breast cancer cells. *Cancer Res* 61:6906–11

Luo ML, Shen XM, Zhang Y *et al.* (2006) Amplification and overexpression of CTTN (EMS1) contribute to the metastasis of esophageal squamous cell carcinoma by promoting cell migration and anoikis resistance. *Cancer Res* 66:11690–9

Marks R (2000) Epidemiology of melanoma. *Clin Exp Dermatol* 25:459–63

Nakayama K, Nakayama N, Davidson B *et al.* (2006) A BTB/POZ protein, NAC-1, is related to tumor recurrence and is essential for tumor growth and survival. *Proc Natl Acad Sci USA* 103:18739–44

Nakayama K, Nakayama N, Wang TL *et al.* (2007) NAC-1 controls cell growth and survival by repressing transcription of Gadd45GIP1, a candidate tumor suppressor. *Cancer Res* 67:8058–64

Patel AM, Incognito LS, Schechter GL *et al.* (1996) Amplification and expression of EMS-1 (cortactin) in head and neck squamous cell carcinoma cell lines. *Oncogene* 12:31–5

Patel AS, Schechter GL, Wasilenko WJ *et al.* (1998) Overexpression of EMS1/cortactin in NIH3T3 fibroblasts causes increased cell motility and invasion *in vitro*. *Oncogene* 16:3227–32

Postovit LM, Seftor EA, Seftor RE *et al.* (2006) Influence of the microenvironment on melanoma cell fate determination and phenotype. *Cancer Res* 66:7833–6

Rodrigo JP, Garcia LA, Ramos S *et al.* (2000) EMS1 gene amplification correlates with poor prognosis in squamous cell carcinomas of the head and neck. *Clin Cancer Res* 6:3177–82

Rothschild BL, Shim AH, Ammer AG *et al.* (2006) Cortactin overexpression regulates actin-related protein 2/3 complex activity, motility, and invasion in carcinomas with chromosome 11q13 amplification. *Cancer Res* 66:8017–25

Satyamoorthy K, Herlyn M (2002) Cellular and molecular biology of human melanoma. *Cancer Biol Ther* 1:14–7

Schuuring E, Verhoeven E, Litvinov S *et al.* (1993) The product of the EMS1 gene, amplified and overexpressed in human carcinomas, is homologous to a v-src substrate and is located in cell-substratum contact sites. *Mol Cell Biol* 13:2891–8

Shen H, Korutla L, Champtiaux N *et al.* (2007) NAC1 regulates the recruitment of the proteasome complex into dendritic spines. *J Neurosci* 27:8903–13

Soengas MS, Lowe SW (2003) Apoptosis and melanoma chemoresistance. *Oncogene* 22:3138–51

Uruno T, Liu J, Zhang P *et al.* (2001) Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat Cell Biol* 3:259–66

van Rossum AG, Gibcus J, van der Wal J *et al.* (2005) Cortactin overexpression results in sustained epidermal growth factor receptor signaling by preventing ligand-induced receptor degradation in human carcinoma cells. *Breast Cancer Res* 7:235–7

Wang GC, Hsieh PS, Hsu HH *et al.* (2009) Expression of cortactin and survivin in renal cell carcinoma associated with tumor aggressiveness. *World J Urol* 27:557–63

Wang X, Cao W, Mo M *et al.* (2010) VEGF and cortactin expression are independent predictors of tumor recurrence following curative resection of gastric cancer. *J Surg Oncol* 102:325–30

Weaver AM, Karginov AV, Kinley AW *et al.* (2001) Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. *Curr Biol* 11:370–4

Weed SA, Du Y, Parsons JT (1998) Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. *J Cell Sci* 111 (Pt 16):2433–43

Wu H, Parsons JT (1993) Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J Cell Biol* 120:1417–26

Xu XZ, Garcia MV, Li TY *et al.* (2010) Cytoskeleton alterations in melanoma: aberrant expression of cortactin, an actin-binding adapter protein, correlates with melanocytic tumor progression. *Mod Pathol* 23:187–96

Yamada S, Yanamoto S, Kawasaki G *et al.* (2010) Overexpression of cortactin increases invasion potential in oral squamous cell carcinoma. *Pathol Oncol Res* 16:523–31

Yang XJ, Gregoire S (2005) Class II histone deacetylases: from sequence to function, regulation, and clinical implication. *Mol Cell Biol* 25:2873–84

Yeasmin S, Nakayama K, Ishibashi M *et al.* (2008) Expression of the bric-a-brac tramtrack broad complex protein NAC-1 in cervical carcinomas seems to correlate with poorer prognosis. *Clin Cancer Res* 14:1686–91

Yuan BZ, Zhou X, Zimonjic DB *et al.* (2003) Amplification and overexpression of the EMS 1 oncogene, a possible prognostic marker, in human hepatocellular carcinoma. *J Mol Diagn* 5:48–53

Zhang X, Yuan Z, Zhang Y *et al.* (2007) HDAC6 modulates cell motility by altering the acetylation level of cortactin. *Mol Cell* 27:197–213

Zhang Y, Zhang M, Dong H *et al.* (2009) Deacetylation of cortactin by SIRT1 promotes cell migration. *Oncogene* 28:445–60