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Molecules and Cells

Twist2 Regulates CD7 Expression and Galectin-1-Induced Apoptosis in Mature T-Cells

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In the periphery, a galectin-1 receptor, CD7, plays crucial roles in galectin-1-mediated apoptosis of activated T-cells as well as progression of T-lymphoma. Previously, we demonstrated that NF-κB downregulated CD7 gene expression through the p38 MAPK pathway in developing immature thymocytes. However, its regulatory pathway is not well understood in functional mature T-cells. Here, we show that CD7 expression was downregulated by Twist2 in Jurkat cells, a human acute T-cell lymphoma cell line, and in EL4 cells, a mature murine T-cell lymphoma cell line. Furthermore, ectopic expression of Twist2 in Jurkat cells reduced galectin-1-induced apoptosis. While full-length Twist2 decreased CD7 promoter activity, a C-terminal deletion form of Twist2 reversed its inhibition, suggesting an important role of the C-terminus in CD7 regulation. In addition, CD7 expression was enhanced by histone deacetylase inhibitors such as trichostatin A and sodium butyrate, which indicates that Twist2 might be one of candidate factors involved in histone deacetylation. Based on these results, we conclude that upregulation of Twist2 increases the resistance to galectin-1-mediated-apoptosis, which may have significant implications for the progression of some T-cells into tumors such as Sezary cells.

INTRODUCTION

CD7 is a glycoprotein identified as a differentiation antigen that is expressed on the surface of peripheral blood T-lymphocytes (Yoshikawa et al., 1993). This protein, which is a member of the immunoglobulin superfamily, was identified as one of the galectin-1 receptors expressed on the surface of most mature T and natural killer cells (Lee et al., 1998). Although it has been reported that CD7 plays a crucial role in T-cell development and activation (Lee et al., 1998), its function is not well understood. The level of surface CD7 expression is dramatically downregulated on the Sezary cell, which is a leukemic form of the cutaneous T-cell lymphoma. As a result, galectin-1-induced apoptosis mediated by CD7 is inhibited in Sezary cells (Pace et al., 2000; Rappl et al., 2002). Downregulation of CD7 is also related to the progression of adult T-cell lymphoma/leukemia, since galectin-3-induced apoptosis is inhibited (Liu et al., 2009). Galectin-3 is also a ligand for CD7 (Fukumori et al., 2003); however, CD7 is not required for the galectin-3-mediated death of Sezary cells (Stillman et al., 2006).

The Twist family of basic helix-loop-helix (bHLH) transcription factors, including Twist1 and Twist2/Dermo-1, are key regulators of mesodermal differentiation (O'Rourke and Tam, 2002) and also play important roles in the epithelial-to-mesenchymal transition involved in cancer metastasis (Yang et al., 2004). Twist2 is known to inhibit the terminal differentiation of a variety of mesodermally derived cell types, including myocytes, osteoblasts and adipocytes (Hebrok et al., 1994; Lee et al., 2003; Spicer et al., 1996; Murray et al., 1992). This protein is also involved in the regulation of the myeloid lineage development (Sharabi et al., 2008). Twist2 can form homodimers or heterodimers with ubiquitously expressed members of E2A bHLH family members (Castanon et al., 2001; Spicer et al., 1996). It acts as a negative regulator of target genes by either binding directly to the E-box consensus motif present in target gene promoters or by associating with transcription factors, such as MEF2, Runx2 and NF-κB, via its C-terminal domain (Bialek et al., 2004; Koh et al., 2008; Sosic et al., 2003; Spicer et al., 1996). Furthermore, Twist2 has been suggested to be involved in histone deacetylation through histone deacetylase (HDAC) activity (Gong and Li, 2002; Lee et al., 2003).

Microarray data showed that Twist1 was highly and selectively expressed in T-cells of patients with Sezary syndrome (van Doorn et al., 2004). Although there is no direct evidence that Twist2 is involved in the progression to Sezary cells, we have shown that this protein downregulates the expression of CD7, which is dramatically reduced in Sezary cells (Koh et al., 2008). Twist2 is a potent negative regulator of gene transcription and is involved in anti-apoptotic processes during tumor progression (Gong and Li, 2002). These results strongly suggest that Twist2 may act as a potential oncogenic transcription factor through the regulation of its downstream target, CD7, in mature T-cells.

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Here, we show that Twist2 is highly expressed in Sezary cells, while CD7 expression is low. The surface level of CD7 was significantly downregulated by ectopic expression of Twist2. Furthermore, chromatin deacetylation through Twist2 was important in the down-regulation of CD7 promoter activity and the consequent reduction of galectin-1-induced apoptosis in mature T-cells.

MATERIALS AND METHODS

Reagents

Mouse anti-human CD7 antibody conjugated with FITC (sc-19606) was purchased from Santa Cruz Biotechnology. FITC-conjugated Annexin V (556547) was obtained from BD Pharmingen. Mouse anti-human Twist2 antibody (ab57997) was purchased from Abcam. ERK pathway inhibitor, PD98059 (P215), and HDAC inhibitors, such as trichostatin A (TSA, T8552) and sodium butyrate (NaB, B5887), were purchased from Sigma.

Cell culture

Jurkat cells, an acute human T-cell lymphoma line, and EL4 cells, a murine T-cell lymphoma line, were grown in RPMI 1640 (Welgene), containing 10% (v/v) fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Hut-78, a Sezary cell line, was purchased from American Type Culture Collection and cultured in IMDM (Hyclone) containing 20% FBS supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Reverse transcription-polymerase chain reaction (RT-PCR)

After T-cells were harvested, total RNA was prepared and used for reverse transcription with SuperScript III (Invitrogen). Sequences for PCR Primers were as follows: human CD7 forward, 5′-TGT CGG ACA CTG GCA CCT-3′; human CD7 reverse, 5′-AGC ACA GTT TCT TTA TCT GT-3′; murine CD7 forward, 5′-TGG CAG ACA CTG GAG ACT AC-3′; murine CD7 reverse, 5′-GAT TCC TTA ATC CCT GAG GC-3′; human Twist2 forward, 5′-AGA AGG GAC AGC AGT GAC ATC G-3′; human Twist2 reverse, 5′-GTG GGA GGC GGA CAT GGA-3´; murine Twist2 forward, 5′-GCT CCA GCT CGC CGG TGT-3′; murine Twist2 reverse, 5′-GTG GGA GGC GGA CAT GGA-3′; human GAPDH forward, 5′-CCT CCA AAA TCA AGT GGG GCG ATG-3´; human GAPDH reverse, 5′-CAT ATT TGG CAG GTT TTT CTA GAC-3′; murine β-actin forward, 5′-CTC TAG ACT TCG AGC AGG AG-3′; murine β-actin reverse, 5′-CCA GAC AAC ACT GTG TTG GC-3′.

Immunoblotting

For immuno-blot analysis, equal amounts of protein from whole-cell extracts were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blotted with anti-CD7 or anti-Twist2 antiserum and secondary antibodies conjugated to horseradish peroxidase. Specific bands were visualized using the ECL system (Pierce).

Flow cytometry and measurement of galectin-1-induced apoptosis

Jurkat and Hut-78 cells were stained with FITC-conjugated anti-CD7 antibody and analyzed with CellQuest™ software using a FACStar Plus (BD Biosciences). A Twist2-expressing construct was transfected into Jurkat cells using Gene Pulse-II RF (Bio-Rad). A total of 5×10^5 cells/100 μl were then treated with the indicated concentration of galectin-1 (Santa Cruz) in 1 mM DTT buffer and were subsequently rocked for 5 h at 37°C. The cells were stained with FITC-conjugated Annexin V (BD Pharmingen) on ice for 15 min and analyzed by flow cytometry. Percent specific apoptosis was calculated using a formula that was previously described (Vacchio and Ashwell, 1997).

Transient transfection and reporter assays

The pCD7-Luc reporter construct containing the CD7 promoter region with basal activity was described previously (Koh et al., 2008). The Twist2-Cdel construct, which encodes a Twist2 box deletion mutant, was cloned by PCR based on a previous report (Gong and Li, 2002). EL4 cells were plated onto six-well plates at 1.0×10^6 cells per well and then transfected with the pCD7-Luc reporter construct plus either full-length Twist-2 or Twist-2-Cdel using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The indicated luciferase reporter construct was co-transfected into EL4 cells with varying amounts of expression plasmids. The pCAGGS control plasmid was used to adjust the total amounts of DNA for each transfection. Twenty-four hours post-transfection, the cells were harvested and their reporter gene activities were determined via luciferase assay (Promega). Luciferase activities were normalized to the total protein concentration as determined by the Bio-Rad protein assay.

RESULTS

Sezary cells show consistently high expression of Twist2 but low expression of CD7

We have previously shown that ectopic expression of NF-κB directly increased CD7 expression and galectin-1-induced apoptosis of immature thymocytes (Koh et al., 2008). However, NF-κB has a controversial role in the survival of mature $CD4^+$ T-cells (Khoshnan et al., 2000) and the CD7 promoter activity was not significantly increased by the addition of NF-κB in mature T-cells (data not shown). Therefore, other factor(s) has crucial role in the regulation of CD7 expression and related apoptosis. The level of Twist2 is low in immature thymocytes unless these cells are activated through TCR, but increased in the mature T-cells (unpublished data). There is a clear correlation between CD7 gene expression and the expression level of Twist2 in immature T-cells, and that Twist2 also downregulates CD7 expression (Koh et al., 2008). To test the possible roles of Twist2 in galectin-1-mediated apoptosis of mature CD4 T-cells and progression of Sezary cells in the periphery, we have investigated the relationship between the expression of Twist2 and CD7.

To better understand the molecular regulatory mechanisms of Twist2 and CD7 in mature T-cells, we measured the expression of Twist2 in Hut-78 Sezary and control Jurkat cells. As shown in Fig. 1A, Twist2 expression was higher in Hut-78 cells compared to Jurkat cells, while CD7 expression was quite low in Hut-78 cells; these results suggested a negative correlation between Twist2 and CD7 expression levels that was consistent with our previous observation in immature T-cells (Koh et al., 2008). The RT-PCR results suggested that the regulation of CD7 expression was controlled at the transcriptional level (Fig. 1A, right panel). Next, we investigated surface levels of CD7 by flow cytometry. In accordance with the data from immuno-blot and RT-PCR assays, Hut-78 cells expressed highly reduced levels of surface CD7 as compared to Jurkat cells (Fig. 1B).

Over-expression of Twist2 inhibits galectin-1/CD7-mediated apoptosis of mature T cells

In order to investigate the effect of reduced CD7 expression in Hut-78 cells on galectin-1-induced cell death, exogenous galectin-1 was administered to Hut-78 and Jurkat cells, and apop-

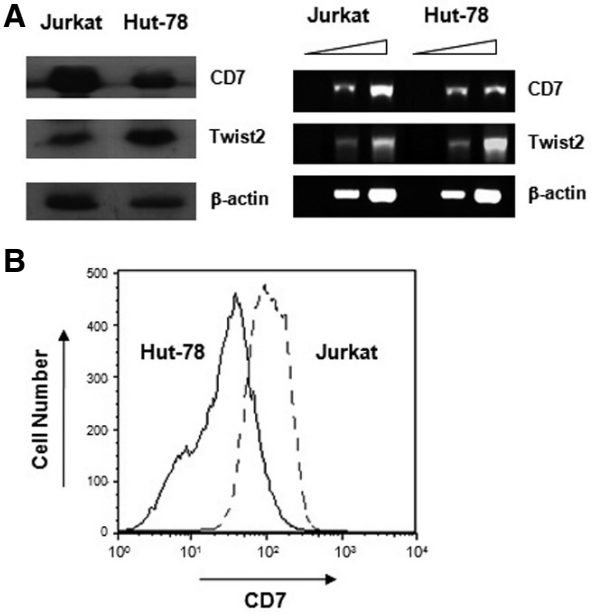

Fig. 1. CD7 expression is downregulated in Hut-78 Sezary cells. (A) Reduction of CD7 expression was confirmed by immunoblot (left panel) and RT-PCR (right panel). In contrast to CD7 expression, Twist2 expression was increased in Hut-78 cells at both transcriptional and translational levels. β-actin was used as a control. (B) The surface level of CD7 on Jurkat and Hut-78 cells was assessed by flow cytometry using an anti-CD7-FITC antibody.

tosis was subsequently assessed by Annexin V staining. As shown in Fig. 2, galectin-1-induced cell death was increased in a dose-dependent manner. However, the level of specific apoptosis was decreased in Hut-78 cells compared to Jurkat cells (about a 10-fold and 15-fold decrease in 20 μg/ml- and 40 μg/ml-treated cells, respectively). This suggested that Hut-78 cells were more resistant to galectin-1-induced cell death than Jurkat cells, which may be attributed to reduced surface levels of CD7.

We also investigated the effects of Twist2 over-expression on galectin-1-induced cell death. The transient transfection of mock or Twist2 over-expression constructs into Jurkat cells resulted in no significant difference in spontaneous apoptosis as measured by Annexin V staining (Fig. 3). When these transfected cells were treated with galectin-1, however, Twist2 over-expression rendered more resistance to galecin-1-mediated cell death, where specific apoptosis was reduced by more than five-fold compared to mock transfectants. This result suggested that Twist2 over-expression inhibited galectin-1-mediated cell death in mature T-cells.

Twist2 directly decreases CD7 promoter activity in mature T-cells

The surface level of CD7 was significantly downregulated in Jurkat cells over-expressing Twist2 (Fig. 4A). However, the manner by which Twist2 controls CD7 expression during T-cell activation and cell death is unclear. The mechanism by which T-cell receptors (TCRs) regulate Twist2 expression is currently controversial; weak TCR signals increased Twist2 expression, while strong TCR signals reduced its expression (data not shown). However, the ERK/MEK pathway, which acts downstream of TCRs, is required for Twist2 expression in mature T-cells. PD98059, an inhibitor of the ERK pathway, significantly reduced Twist2 expression rather than increasing CD7 expression (Fig. 4B).

In a previous report, we showed that Twist2 inhibited NF-κB-mediated induction of CD7 promoter activity through binding to the E-box in immature T-cells (Koh et al., 2008). In order to investigate whether Twist2 can repress CD7 expression in mature T-cells, a reporter gene construct containing the CD7 proximal promoter region was co-transfected into EL4 cells, the mature T-cell lymphoma cell line, along with the expression vectors for intact Twist2 or Twist2-Cdel, the C-terminal deletion form of Twist2. The C-terminal region of Twist2 has been reported to function in transcriptional repression by interacting with other transcription factors, including NF-κB and Runx2 (Gong and Li, 2002). While CD7 promoter activity was downregulated by exogenous over-expression of Twist2, it was increased by over-expression of Twist2-Cdel (Fig. 5A). This result suggested that the C-terminal region was important for Twist2-mediated repression of CD7 promoter activity, and that Twist2-Cdel may act as a dominant-negative mutant.

Chromatin acetylation is important for regulation of CD7 promoter activity

Remarkably, exogenous Twist2-Cdel showed much higher CD7 promoter activity than the basal level seen in control cells. This may be related to the role of Twist2 in chromatin remodeling; Twist2 is known to be involved in histone deacetylation in cooperation with HDACs and in transcriptional repression (Gong and Li, 2002; Lee et al., 2003). In addition, Twist2 was shown to bind to HDAC members through immunoprecipitation

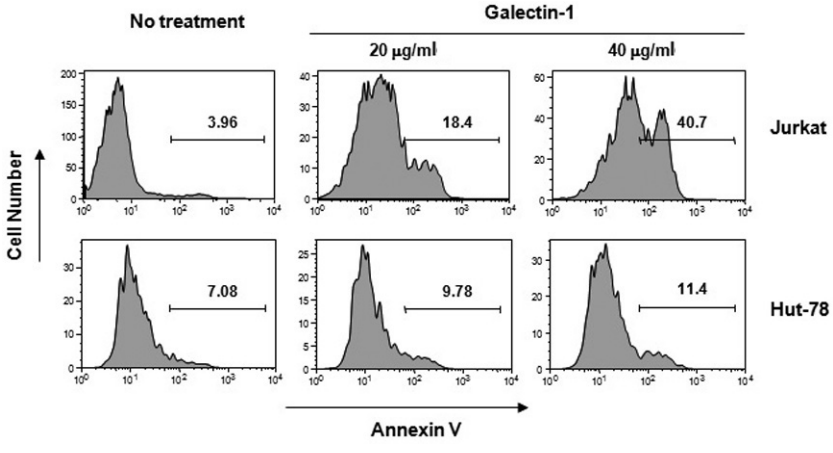

Fig. 2. Sezary cells are more resistant to galectin-1-mediated T-cell death. Galectin-1 treatment induced the apoptosis of T-cells in a dose-dependent manner. A significantly reduced sensitivity to galectin-1-mediated apoptosis was observed in Hut-78 cells compared to Jurkat cells. The cells were treated with increasing amounts of galectin-1 (0, 20 and 40 μg/ml) for 5 h at 37°C. Apoptotic cells were stained with Annexin V-FITC and analyzed by flow cytometry.

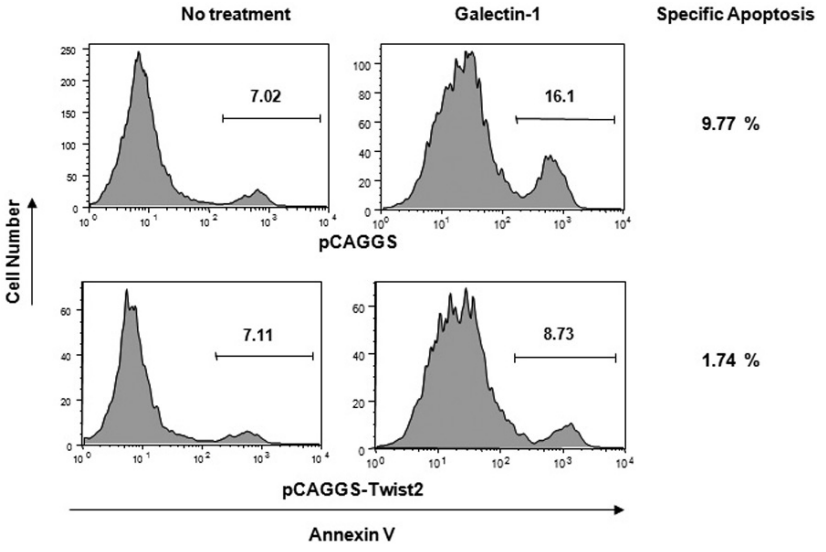

Fig. 3. The sensitivity to galectin-1-mediated T-cell death is reduced by ectopic expression of Twist2. Jurkat cells were transfected with a mock or Twist2 expression vector and were subsequently incubated for 48 h. These cells were then treated with 2 μg of galectin-1 for 5 h at 37°C, followed by Annexin V-FITC staining. Apoptotic cell death was analyzed by flow cytometry. Specific apoptosis was indicated in the right panel.

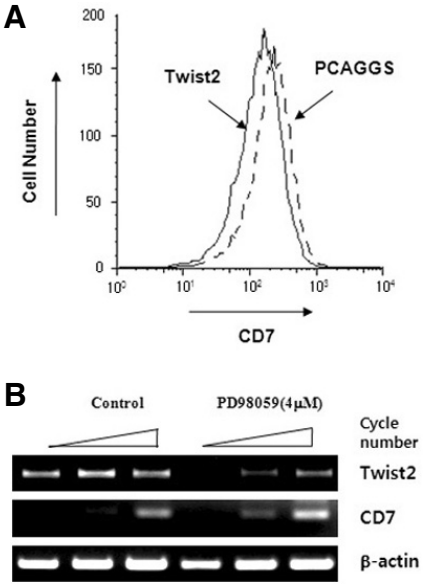

Fig. 4. Twist2 downregulates CD7 expression in mature T-cells. (A) Jurkat cells were transfected with a Twist2 expression vector or a control vector, and surface CD7 expression was then analyzed as described in Fig. 1. (B) Blocking the ERK/MEK signaling pathway prevents Twist2 gene expression, while increasing CD7 expression. EL4 cells were treated with PD98059, an inhibitor of the ERK pathway, or DMSO as a negative control, for 24 h and total RNAs were then prepared from these cells. Semi-quantitative RT-PCR was performed using specific primers as indicated. β-actin was used as an internal control.

(unpublished data). To investigate whether CD7 expression is regulated by histone acetylation, the level of CD7 expression was determined after treatment with TSA and NaB, which are HDAC inhibitors. As shown in Fig. 5B, either TSA or NaB significantly induced the CD7 expression, suggesting that HDAC is involved in the regulation of normal expression of CD7 through chromatin remodeling. These results suggested that CD7 expression is regulated by histone acetylation, possibly through HDAC and Twist2.

DISCUSSION

Previous reports demonstrated that Twist1 and Twist2 expression was regulated by specific proliferating signals, including IGF-1, TGF-β and EGF, and was also involved in the anti-apoptotic process (Dupont et al., 2001; Lo et al., 2007; Murakami et al., 2008). Additionally, Twist1 was upregulated by EGF receptors through the STAT3 pathway during tumor progression (Lo et al., 2007). Meanwhile, the regulatory mechanisms of Twist2 expression are not well understood. Twist1 gene expression was shown to be specifically increased in Sezary syndrome patients (van Doorn et al., 2004), but there is no evidence that Twist2 is also involved in the progression of Sezary cells.

In this paper, we have shown that Twist2 is highly expressed in Hut-78 Sezary cells, while CD7 expression is low, and that these cells are consequently more resistant to galectin-1-mediated cell death. Over-expression of Twist2 in mature T-cells inhibits galectin-1-mediated cell death by reducing the surface expression of CD7. Furthermore, the regulation of CD7 promoter activity is dependent on histone acetylation/deacetylation. In this case, the C-terminal domain of Twist2 may be important in maintenance of the inactivated state of chromatin by interacting with HDACs.

The cAMP response element binding protein (CREB) plays an important role in regulating associated gene expression via the cAMP response element. In a previous report, we showed that H-89, an inhibitor of the MSK1-CREB pathway, significantly reduced CD7 promoter activity (Koh et al., 2008). CREB activity can be also disrupted by expression of a dominant negative form of CREB (A-CREB), which inhibits the DNA binding and function of CREB family members. After CREB has been phosphorylated by Protein Kinase A, it stimulates gene transcription by recruiting the adaptor molecule, CREB-binding protein (CBP). Interestingly, A-CREB dramatically repressed CD7 promoter activity in our recent work (data not shown), which confirmed our previous results showing that the CREB pathway was important for CD7 expression (Koh et al., 2008).

In addition to its role in recruiting elements of the basal transcription apparatus, CBP has histone acetyl transferase (HAT) activity, which leads to addition of acetyl groups on histones (Bannister and Kouzarides, 1996). Since histones are general repressors of gene activity (Kwon and Workman, 2008), their

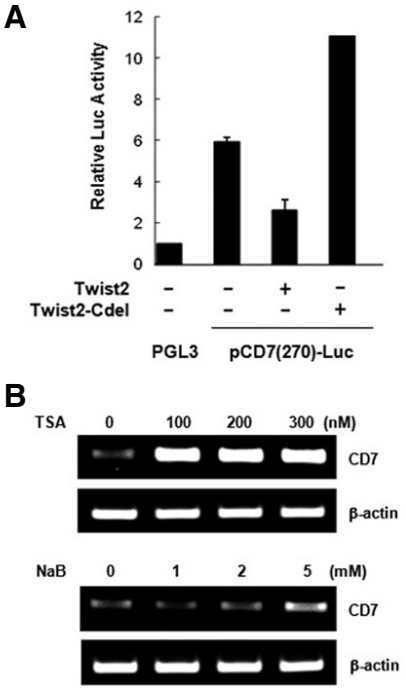

Fig. 5. Histone acetylation is important for CD7 promoter activity. (A) Twist2 reduced CD7 promoter activity. The pCD7(-270)-Luc construct containing the basal CD7 promoter region was co-transfected along with the Twist2 or the Twist2-Cdel expression vector into EL4 cells. Twist2-Cdel encodes a C-terminal deletion mutant of Twist2. pGL3 is a control vector without the CD7 promoter. The relative activity to the pGL3 control was shown as the mean ± standard error (SE). (B) Treatment of HDAC inhibitors increased CD7 expression. EL4 cells were incubated in the presence of TSA (0 to 300 nM) or NaB (0 to 5 mM) for 24 h. Semi-quantitative RT-PCR was performed using specific CD7 primers as indicated. β-actin was used as an internal control.

modification tends to inhibit the repression of target gene expression. A-CREB-related inhibition of HAT activity may be explained by this reduced CD7 promoter activity. Therefore, we cannot preclude the possibility that Twist2 inhibits HAT activity as in the case of Twist1 in TGF-β signaling (Qiu et al., 2006). It was also reported that the N-terminal domain of Twist1 interacts directly with two HATs, including p300 and p300/CREB binding protein-associated factor (PCAF), and inhibits their HAT activities (Hamamori et al., 1999). However, in the case of Twist2, the C-terminal domain is more important in repressing expression of the target gene (Gong and Li, 2002).

Taken altogether, our results suggest that Twist2 is one of the main regulators of CD7 levels in mature T-cells, and the failure of Twist2 regulation may result in the generation of abnormal T-cell populations such as Sezary cells.

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