이학박사학위논문

전사조절 인자인 Twist2에 의한 흉선 T세포의

부정적 선별과정에 관한 연구

Studies on the function of a transcription factor

Twist2 on negative selection of thymocytes

2016년 8월

서울대학교 대학원

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이 논문을 이학박사 학위논문으로 제출함

2015년 11월

서울대학교 대학원

생명과학부

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오상욱의 이학박사 학위 논문을 인준함

2015년 12월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

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Submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

to the faculty of the

School of Biological Sciences

at

SEOUL NATIONAL UNIVERSITY

by

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Date Approved:

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ABSTRACT

Studies on the function of a transcription factor Twist2 on negative selection of thymocytes

Sangwook Oh

Self-reactive T cells are eliminated by negative selection during thymocyte development. Thymocytes bearing high-affinity T-cell receptor (TCR) to self-antigen induce *Nur77* and *N*or-1 expression and undergo apoptosis. Even though the regulation of *Nur77* and *N*or-1 expression has been well studied, a factor(s) repressing both *Nur77* and *Nor-1* in surviving thymocytes has not yet been identified. In addition, the disruption of *Hdac7* gene, a repressor of *Nur77* expression, failed to derepress the *Nur77* expression. These suggest an existence of an additional and as yet unidentified factor(s) that controls the expression of *Nur77* and *Nor-1* during thymocyte selection. Twist2, a basic helix-loop-helix protein, suppressed negative selection of thymocytes by repressing both *Nur77* and *Nor-1* expression. Twist2 formed a repressive complex on the *Nur77* promoter by interacting with HDAC7, which compete out the recruitment of p300, the co-activator. The expression of *Twist2* was differentially regulated depending on the strength of TCR signaling. In particular, *Twist2* expression was down-

regulated in negatively-selected thymocytes. Therefore, this study shows that TCR-dependent Twist2 expression determines the fate of thymocytes during thymic selection.

Keywords: Twist2, Nur77, Nor-1, Thymocytes, Negative selection, Apoptosis, HDAC7

Student Number: 2007-20349

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I. T cell development in the thymus

I-1. Importance of thymic selection processes

Bone-marrow-derived T cell progenitors migrate into the thymus, and then develop and differentiate into mature T cells. T cells recognize foreign antigens through their own T cell receptors (TCRs). TCRs are produced by somatic recombination on early stage of thymocyte development and differentiation in order to recognize various antigens. However, this kind of TCR diversity is associated with possible risks of immune responses to self-antigens. In order to resolve TCR-mediated unwanted autoreactivity, our body has to develop a strategy to maintain T cell diversity and avoid autoreactivity at the same time. T cells develop in the thymus and self-reactive T cells are eliminated by thymic negative selection. Therefore, precise regulation of thymic negative selection is required for the balance between the diversity and the autoreactivity in our immune system.

I-2. TCR rearrangement during thymocyte development

Thymocyte developmental stages can be divided into three major parts,

DN (double negative), DP (double positive), and SP (single positive) stages, respectively, by their surface expression of CD4 and CD8 co-receptors. DN stage cells express neither CD4 nor CD8 co-receptors. DN stage cells differentiate into DP stage cells that express both CD4 and CD8 co-receptors. And, then, DP stage cells differentiate into SP cells that express either CD4 or CD8 co-receptors. DN stage cells further are dissected into four sub-stages using CD44 and CD25 expression; DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$), and DN4 ($CD44^{-}CD25^{-}$). At DN3 stage, TCR (T cell receptor) β chain of thymocytes are randomly rearranged and DN3 stage cells bearing functional TCR β chain on their surface further differentiate into DN4 stage cells. This selection process is called β-selection. Through the DN4 to early DP stages, cells are clonally expanded and TCR α chain genes are randomly rearranged. Eventually, rearranged TCR α and β chains form heterodimers on the surface of thymocytes at DP stage.

I-3. The affinity between TCR and MHC determines the fate of thymocytes

TCRs on the surface of thymocytes recognize peptides on the major histocompatibility complexes (pMHCs) of antigen presentation cells (APCs) and transduce TCR signal. In the periphery, T cells recognize pathogenic peptide presented on the APCs via their TCRs and trigger immune responses. Thus, if peripheral T cells had self-peptide responsible TCRs on their surface,

it would be possible to attack the host and cause the autoimmune diseases. This fatal risk is avoided by the selection processes that occur in the thymus. T cells bearing TCRs that transduce weak and intermediate TCR signal-strength undergo positive selection and further differentiate into SP cells. On the contrary, T cells bearing TCRs that transduce strong TCR signal-strength undergo negative selection and die by activation-induced cell death (AICD). Consequently, only T cells that could recognize pMHCs, but failed to overreact to self-peptide-loaded MHCs (self-pMHCs) can survive and keep the immunological tolerance in our immune system.

II. Positive and negative selection of thymocytes

At the DP stage of thymocyte development, DP thymocytes express only one type of TCRs on their surface. Though the fate of thymocytes are completely different depending on their TCR-peptide-MHC affinity, both positive and negative selections are induced by TCR signaling. Though both two selection processes are mediated by TCR signaling, the molecular mechanisms that control each selection processes are different (Fu and others, 2014). Hence, how thymocytes discriminate the quality and quantity of TCR signaling is very important to understand the thymocyte development.

II-1. Positive selection of thymocytes

In the early studies, positive selection is thought as a passive selection

that just not undergoing negative selection. This model suggested that T cells bearing functionally competent TCRs are survive and mature into peripheral T cells unless these T cells are self-reactive and actively eliminated in the thymus.

However, successful rearrangement of TCR α and β chains are not sufficient to complete positive selection. To survive and undergo further differentiation, T cells should interact with pMHCs on APCs. Pro-survival (or anti-apoptotic) molecules such as Bcl-2, Bcl-X_L, and Mcl-1 are required for the survival of positively-selected thymocytes because lack of these molecules promote cell death of DP thymocytes regardless of TCR signaling (Dzhagalov and others, 2008; Gratiot-Deans and others, 1993; Gratiot-Deans and others, 1994; Grillot and others, 1995; Veis and others, 1993). Positive selection occurs at the cortical area of thymus. During DN to DP transition, up-regulation of Bcl- X_L expression is necessary for the survival. During positive selection, Bcl-X_L is down-regulated. However, Bcl-2 expression is up-regulated for the compensation of the down-regulation of Bcl-X_L during positive selection.

II-2. Negative selection of thymocytes

To prevent autoimmunity in our immune system, self-reactive T cells should be eliminated or suppressed. The former is the negative selection of thymocytes and the latter is the generation of regulatory T (T_{reg}) cells. In other

words, self-tolerance consist of negative selection ‘central tolerance’ and the suppressive function of T_{reg} cells called ‘peripheral tolerance’ (Mathis and Benoist, 2004; Xing and Hogquist, 2012).

Unlike positive selection exclusively occurs at the cortical area of thymus, negative selection occurs at the both cortical and medullary area of thymus (Kappler and others, 1987; Kisielow and others, 1988; Sha and others, 1988). This is quite rationale because DP cells, which undergo both positive and negative selection, are located in the cortex, while SP cells, which are already positively-selected and undergo negative selection, are located in the medulla (Gill and others, 2003; Petrie, 2003; van Ewijk, 1991). Accordingly, if immature thymocytes recognized self-peptides and showed responsiveness, those cells would undergo negative selection in any area of thymus. Self-responsiveness is determined by the affinity between TCRs of T cells and pMHCs on APCs. Depending on the affinity between TCRs and MHCs, the quality and the quantity of TCR signaling is different (Zikherman and Au-Yeung, 2015). Unlike intermediate affinity between TCR and pMHC up-regulates pro-survival molecules, high affinity between TCR and pMHC up-regulates pro-apoptotic molecules (Sohn and others, 2007). Consequently, self-reactive T cells bearing TCRs that show high affinity with pMHCs are eliminated.

III. Regulation of TCR-mediated apoptosis of thymocytes

III-1. The role of NR4A receptor family members in thymic negative selection

In the early 1990's, NR4A receptor family members are identified as an immediate early gene activated by TCR signal and induce apoptosis of thymocytes (Liu and others, 1994; Woronicz and others, 1994; Woronicz and others, 1995). NR4A receptor family consists of three members, Nur77 (NR4A1), Nurr1 (NR4A2), and Nor-1 (NR4A3), respectively. Among the three members, only Nur77 and Nor-1 was induced in thymocytes after TCR stimulation (Cheng and others, 1997). Enforced expression of Nur77 specifically in thymocytes of mice caused massive cell death of thymocytes, which reflects the crucial function of Nur77 in thymocyte AICD (Calnan and others, 1995). However, *Nur77*-null mice exhibited normal thymocyte development in both positive and negative selection (Lee and others, 1995). Later, these unexpected results were due to the functional redundancy between Nur77 and Nor-1 (Cheng and others, 1997). Massive cell death of thymocytes was detected in *Nor-1* transgenic mice as well as *Nur77* transgenic mice (Cheng and others, 1997). Transgenic mice expressing dominant negative-form of Nur77 showed abnormal negative selection because dominant negative-form of Nur77 protein interferes Nor-1 function as well as Nur77 function (Calnan and others, 1995).

III-2. Nur77 is induced by TCR signaling and causes TCR-mediated apoptosis of thymocytes

After the crucial role of Nur77 in negative selection was emerged, several groups investigated the regulation mechanism of *Nur77* expression during T cell activation (Woronicz and others, 1995; Youn and others, 2000; Youn and Liu, 2000). There are two *in vitro* experimental systems to mimic TCR stimulation. One uses anti-CD3 antibody and the other uses chemical reagents, PMA (phorbol 12-myristat 13-acetate) and ionomycin (Ca^{2+} ionophore). Though TCRs are membrane receptors that capture extracellular signal, they lack the intracellular domain to transduce signal. Instead, CD3 complexes are recruited to immunological synapse where TCRs encounter with pMHCs and immunoreceptor tyrosine-based activation motifs (called ITAM) of CD3 complex are phosphorylated (Weiss and Littman, 1994). Hence, direct activation of CD3 complex using anti-CD3 antibody could mimic the TCR signaling itself.

Transduced TCR signal activates two signaling pathways, PKC and Ca^{2+} pathway, respectively, though the TCR signaling cascades are much more complicated than this (Werlen and Palmer, 2002). For these reasons, chemical reagents that activate PKC (by PMA) and Ca^{2+} (by ionomycin) pathways could mimic TCR signaling *in vitro* system (Shao and others, 1999; Wilkinson and Kaye, 2001). By dissection the TCR signal-pathways, it was revealed that Ca^{2+} signal is critical for the induction of *Nur77* expression (Liu and others, 1994).

However, though Ca^{2+} pathway sufficiently induced *Nur77* expression, Ca^{2+} pathway alone could not cause the apoptosis of thymoma and thymocytes (Liu and others, 1994). To induce apoptosis of thymocytes, Nur77 protein migrates to the mitochondria and interacts with BH3-domain, and then triggers the releasing of cytochrome c, which activates caspase-dependent programmed cell death (Stasik and others, 2007; Thompson and others, 2010).

III-3. Regulation mechanism of *Nur77* expression after TCR stimulation

Among the cis-elements on the *Nur77* promoter, it was reported that the MEF2 (Myogenic enhancer factor-2) site was responsible to TCR stimulation (Blaeser and others, 2000; Woronicz and others, 1995; Youn and others, 2000; Youn and Liu, 2000). The MEF2 site on the *Nur77* promoter was named after the binding protein at this site, MEF2D (Woronicz and others, 1994; Woronicz and others, 1995; Youn and Liu, 2000). MEF2D was identified as a transcriptional activator that induces *Nur77* expression. However, MEF2D was constitutively bound to the MEF2 site regardless of TCR signaling (Youn and Liu, 2000; Youn and others, 1999). It implies the presence of another transcriptional regulator(s) in *Nur77* expression depending on the TCR signaling. Cabin-1 was reported as a transcriptional repressor in *Nur77* expression (Youn and Liu, 2000; Youn and others, 1999). Cabin-1 interacted with MEF2D and suppressed *Nur77* induction after TCR stimulation. However, the mice expressing truncated-mutant of Cabin-1, which lacks the binding domain with MEF2D, displayed normal thymocyte development

(Esau and others, 2001).

Another candidate molecule that represses *Nur77* expression was reported in 2003 (Dequiedt and others, 2003). This group showed that histone deacetyltransferase 7 (HDAC7) repressed *Nur77* expression and AICD of thymoma after TCR stimulation. The expression of HDAC7 was high at DP cells and HDAC7 interacted with MEF2D in TCR unstimulated thymocytes. TCR signal activates protein kinase D1 (PKD1) and activated PKD1 phosphorylates HDAC7 (Dequiedt and others, 2005; Parra and others, 2005). Phosphorylated HDAC7 exported to the cytoplasm and empty *Nur77* promoter replaced by transcriptional activators such as NFATp and p300 (Blaeser and others, 2000; Youn and others, 2000). It was also reported that phosphorylated HDAC7 in the cytoplasm was dephosphorylated by Myosin phosphatase and transported back to the nucleus (Parra and others, 2007). Mutant mice in the phosphorylation sites of HDAC7, which lack the ability to export cytoplasm after TCR stimulation, displayed abnormalities in negative selection of thymocytes (Kasler and others, 2012). Thus, nuclear/cytoplasmic shuttling of HDAC7 is suggested to explain the TCR signal-dependent *Nur77* expression.

III-4. Unresolved questions in *Nur77* regulation

Though HDAC7 sheds light on the *Nur77* regulation mechanism, there was still remained some unresolved questions. The first one is *Nur77*

expression was not induced in the absence of HDAC7 in DP thymocytes (Kasler and others, 2011). If binding of HDAC7 on the *Nur77* promoter by interacting with MEF2D was critical for the repression of *Nur77* expression in TCR unexperienced thymocytes, the absence of HDAC7 could increase the expression of *Nur77* because the treatment of trichostatin A (TSA), a pan-HDAC inhibitor, induces *Nur77* expression without TCR stimulation (Dequiedt and others, 2003). However, *Nur77* expression in thymocytes of HDAC7 knockout mice was comparable with that of control mice. Thus, these data suggest that there should be another repressor in addition to HDAC7.

The second one is that nuclear export of HDAC7 is mediated by PKC pathway (Dequiedt and others, 2005; Parra and others, 2005). As mentioned above, TCR signaling can be mimicked by stimulating two signal pathways, PKC and Ca^{2+}, respectively, and Ca^{2+} pathway is mainly responsible for the induction of *Nur77* expression (Liu and others, 1994). Accordingly, though nuclear/cytoplasmic shuttling model of HDAC7 is acceptable to explain the regulation mechanism of *Nur77* induction after TCR stimulation including both PKC and Ca^{2+} pathways, it is not sufficient to explain the Ca^{2+} pathway-dependent *Nur77* induction.

The last one is that nuclear/cytoplasmic shuttling model of HDAC7 could not explain the differential expression pattern of *Nur77* depending on the TCR signal-intensity. The expression of *Nur77* shows positive correlation with TCR signal-intensity. In particular, the expression level of *Nur77* in

negatively-selected thymocytes is much higher than that in positively-selected thymocytes. Unlike the differential expression pattern of *Nur77* depending on TCR signal-strength, nuclear exportation of HDAC7 happens even in the positively-selected thymocytes (Kasler and others, 2011). Hence, HDAC7 itself is not sufficient to explain the fine regulation of *Nur77* expression depending on TCR signal-strength.

VI. Aim of this study

Nevertheless the importance of differential regulation in *Nur77* expression depending on TCR signal-strength, the regulation mechanism is not heretofore unclear. Furthermore, the mechanism that simultaneously regulates both *Nur77* and *Nor-1* expressions is also still remained unidentified. Understanding how *Nur77*/*Nor-1* is differentially expressed between positive and negative selections serves to provide the precise mechanism how TCR signal is translated into functional molecules.

The bHLH transcription factors generally play critical roles in cell lineage determination, proliferation and differentiation through direct binding to the E-box or interaction with other transcription factors (Cserjesi and others, 1995; Lister and Baron, 1998; Murre and others, 1989). Twist2, a member of bHLH family, has been known to inhibit the differentiation of myocytes, osteoblasts, and adipocytes (Hebrok and others, 1994; Lee and others, 2003; Murray and others, 1992; Spicer and others, 1996). In addition, it is reported

that Twist2 regulates immune cell development and mature T cell apoptosis (Koh and others, 2009; Sharabi and others, 2008). To regulate the transcription of other genes, Twist2 can form homo- or heterodimer with other bHLH members to bind the E-box consensus motif present in target gene promoter (Castanon and others, 2001; Spicer and others, 1996). During myogenesis, Twist2 inhibited the transcriptional activity of MyoD by physical interaction with MEF2C (Gong and Li, 2002). In this case, the inhibition of MEF2C activity by Twist2 was not mediated through the E-box because there was no conserved E-box sequence, but rather Twist2 repressed MyoD activity through protein-protein interaction with MEF2C. Furthermore, it has been thought that Twist2 could recruit HDACs to promoter for the suppression of target gene expressions (Gong and Li, 2002; Koh and others, 2009; Lee and others, 2003).

To reveal the possible roles of Twist2 in thymocyte development, the physiological significances in both Twist2 conditional knockout mice and Twist2 transgenic mice are investigated. *Twist2* was specifically induced in post-selected DP thymocytes by the positive selection-strength of TCR signaling. However, the *Twist2* induction is discontinued in thymocytes undergoing negative selection. In correspondence with the expression pattern, the TCR-induced apoptosis was augmented in thymocytes of *Twist2* conditional knockout mice, whereas transgenic expression of *Twist2* inhibited TCR-mediated apoptosis of thymocytes. Twist2 facilitated the formation of a repressive complex on the *Nur77* and the *Nor-1* promoter by recruiting

HDAC7 and competing out p300. Therefore, these data suggest that Twist2 is differentially regulated depending on the strength of TCR signal and regulates *Nur77* and *Nor-1* expression. Eventually, Twist2 controls the fate of thymocytes by discriminating the strength of TCR-signal.

MATERIALS AND METHODS

Mice

A cDNA encoding mouse *twist2* was obtained by reverse transcription-PCR (RT-PCR) from thymocyte RNA preparations and was cloned into the BamHI site of the p1017 vector under the control of a mouse *Lck* proximal promoter by using conventional cloning procedures. To specifically delete *twist2* in thymocytes, *twist2*$^{f/f}$ mice were crossed with *Cd4*-Cre-Tg mice. *Tcrα*$^{-/-}$, OT-II TCR-Tg, and Rip-mOVA-Tg mice were purchased from Jackson Laboratory. *Rag*$^{-/-}$ X DO11.10 TCR-Tg mice were purchased from Taconic Farm. The mice were bred and maintained under specific pathogen-free condition, and experiments were performed in accordance with institutional and national guidelines.

Flow cytometry analysis and cell sorting

Thymocytes were harvested, stained, and analyzed on a FACSCanto II (BD Biosciences). Abs with the following specificities were used for staining: anti-CD69-PerCP, anti-Vα2-biotin, anti-TCRβ-APC, anti-CD4-PE, anti-CD4-PEcy7, anti-CD8α-APCcy7, anti-CD24-PE-cy7, anti-CD24-FITC, anti-DO11.10 TCR-PerCP, anti-Nur77-PE and anti-Streptavidin-APC (all from BD Biosciences or eBioscience). Where indicated, thymocytes were sorted into

$CD4^+CD8^+TCR\beta^{lo}CD69^{lo}$ (preDP) and $CD4^+CD8^+TCR\beta^{int/hi}CD69^{hi}$ (postDP) subpopulations on a FACSAria II (BD Biosciences). The purity of all sorted population was above 95%. Untouched B cells were sorted using Dynabeads (Invitrogen) following manufacturer's instruction. Briefly, splenocytes of BALB/c mice were mixed with Dynabeads and biotin-conjugated anti-CD4, anti-CD8, anti-Ter119, anti-CD3ε, anti-CD11b, and anti-GR1 antibodies for 20 min at RT. Supernatants containing bead-free untouched B cells were separated using magnet and collected into a new tube. DP cell viability was detected by gating DO-$TCR^+CD4^+CD8^+$ population after incubation for 24 hr with untouched B cells. $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) peptide (GL Biochem, Shanghai) was preloaded 30 min to untouched B cells before the incubation with thymocytes. For AnnexinV staining, thymocytes and 16610D9 cells were activated with PMA and ionomycin and apoptosis was detected using AnnexinV-FITC apoptosis kit (BD Biosciences) according to manufacturer's protocol. For intracellular staining, fleshly isolated thymocytes were stained with anti-CD4-PEcy7, anti-CD8-APCcy7, anti-CD24-FITC, anti-Vα2-Biotin and anti-Streptavidin-APC antibodies. Stained thymocytes were fixed and further stained with anti-Nur77-PE antibody using Foxp3 Fix/Perm kit (eBioscence) following manufacturer's instruction.

Cell culture and luciferase assay

Primary thymocytes were cultured on RPMI medium with 10% FBS and 2-

mercaptoethanol. 16610D9 cells were maintained with Opti-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 2-mercaptoethanol. For luciferase assay, the NUR77 and the NOR-1 promoters originated from genomic DNA of Jurkat cell line and the twist2 promoter originated from genomic DNA of c57BL/6 mouse were cloned into pGL3-basic vector. Point mutations in each promoter constructs were generated by site-directed mutagenesis as instructed (Stratagene, La Jolla, CA).

Semi-quantitative and quantitative PCR

RNA was extracted from the sorted populations or cells by Trizol reagent (Invitrogen) and then used for reverse transcription. Quantitative PCR (qPCR) was conducted by using Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific) with indicated primers.

Chromatin immunoprecipitation assay and co-immunoprecipitation assay

For chromatin immunoprecipitation (ChIP) assay, sample preparation from thymocytes and 16610D9 cells and immunoprecipitation were performed using anti-Twist2 (3C8, Abnova), anti-HDAC7 (H-273x, Santa Cruz), and anti-p300 (C-20, Santa Cruz) antibodies as previously described (Choi and others, 2015). DNA was purified with a QIAquick Spin kit (Qiagen) and eluted

DNA was analyzed by PCR with indicated primers (Table 1). Quantification of the PCR band intensity was calculated using ImageJ software (National Institutes of Health, Bethesda, Maryland). For co-immunoprecipitation (co-IP) assay, 293T cells were harvested and whole cell extracts were disrupted with lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM MgCl2, 1 mM EDTA, and 1% Triton X-100) containing protease inhibitors cocktail (Roche), 10 mM NaF, 10 mM sodium pyrophosphate, and 100 mM sodium vanadate. Western blotting was performed as described previously.

Bimolecular fluorescence complementation (BiFC) analysis

The principle of BiFC was described elsewhere. Briefly, HDAC7, MEF2D, Twist2 and the C-terminal deleted form of Twist2 (Twist2CΔ) were inserted into either VN-173 or VC-155 vector. 293T cells were transfected with a combination of VN/VC vectors. Fluorescence was detected by either fluorescence microscope (LSM 710) or FACSCanto II.

Statistical analysis

Statistical analysis was performed using Prism 5.0b (GraphPad software). Two-tailed Student t test were used to compare the statistical significance of differences between the samples and their respective controls. The P values are represented in the figures by asterisks (*P<0.05, **P<0.01, and

***P<0.001). The absence of an asterisk or the mark of ns (non-significant) indicates that the change relative to control is not statistically significant.

RESULTS

The expression pattern of Twist2 during thymocyte development

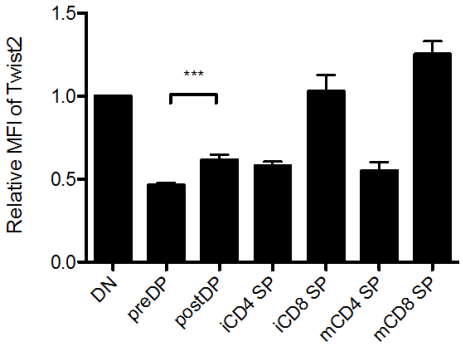
To determine the roles of Twist2 during thymocyte development, total thymocytes was intracellular stained with anti-Twist2 monoclonal antibody and the mean fluorescence intensity of Twist2 was calculated in each thymocyte subpopulations after gating with TCRβ, CD69, CD4, and CD8. In this gating strategy, thymocyte developmental stages can be divided in an order: $TCR\beta^{lo}CD69^{lo}CD4^{-}CD8^{-}$ (DN), $TCR\beta^{lo}CD69^{lo}CD4^{+}CD8^{+}$ (preDP), $TCR\beta^{int/hi}CD69^{hi}CD4^{+}CD8^{+}$ (postDP), $TCR\beta^{int/hi}CD69^{hi}CD4^{+}CD8^{-}$ (immature CD4 single positive; iCD4 SP) and $TCR\beta^{int/hi}CD69^{hi}CD4^{-}CD8^{+}$ (iCD8 SP), and $TCR\beta^{hi}CD69^{lo}CD4^{+}CD8^{-}$ (mature CD4 SP; mCD4 SP) and $TCR\beta^{hi}CD69^{lo}CD4^{-}CD8^{+}$ (mCD8 SP). The remarkable features in Twist2 expression pattern is down-regulation during DN to preDP transition and up-regulation during preDP to postDP transition (Figure 1). Further study was revealed that Twist2 expression was high at DN4 stage where pre-TCR signal is transduced and thymocytes escaping β-selection is expanded by vigorous proliferation (Figure 2).

The second feature in Twist2 expression pattern is also related with

Figure 1. Twist2 expression pattern during thymocyte development.

Thymocytes from C57BL/6 mice were stained with anti-TCRβ, anti-CD69, anti-CD4, and anti- CD8α antibodies. Stained cells were fixed using fix/perm kit (eBioscience) following manufacturer's instruction. Expression of Twist2 was detected by FACSCanto II after staining with anti-Twist2 antibody. Mean fluorescence intensity of Twist2 in each thymocyte subsets were normalized to the results of DN population (error bars, ±SEM).

DN; $TCR\beta^{lo}CD69^{lo}CD4^{-}CD8^{-}$, preDP; $TCR\beta^{lo}CD69^{lo}CD4^{+}CD8^{+}$, postDP; $TCR\beta^{int/hi}CD69^{hi}CD4^{+}CD8^{+}$, iCD4 SP; $TCR\beta^{int/hi}CD69^{hi}CD4^{+}CD8^{-}$, iCD8 SP; $TCR\beta^{int/hi}CD69^{hi}CD4^{-}CD8^{+}$, mCD4 SP; $TCR\beta^{hi}CD69^{lo}CD4^{+}CD8^{-}$, and mCD8 SP; $TCR\beta^{hi}CD69^{lo}CD4^{-}CD8^{+}$


Relative MFI of Twist2
1.5
1.0
0.5
0.0
***
DN
preDP
postDP
iCD4 SP
iCD8 SP
mCD4 SP
mCD8 SP

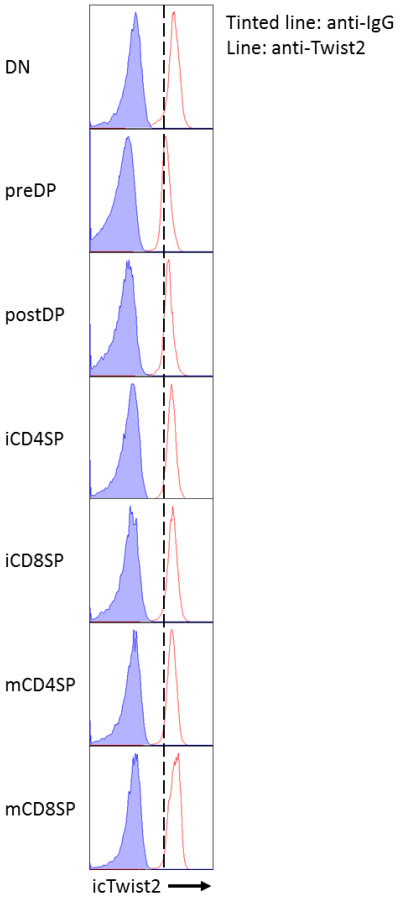
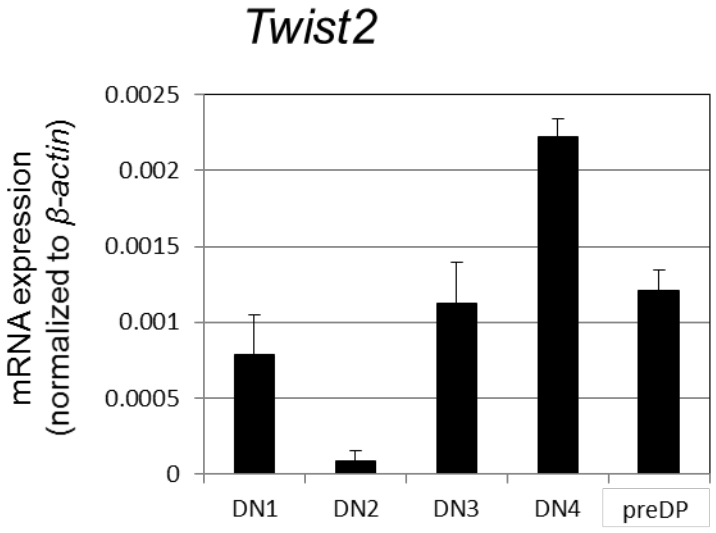

Tinted line: anti-IgG
Line: anti-Twist2
DN
preDP
postDP
iCD4SP
iCD8SP
mCD4SP
mCD8SP
icTwist2

Figure 2. Twist2 expression pattern in DN subsets of thymocytes

Thymocyte subsets from C57BL/6 mice were sorted and Twist2 expression was detected in each subsets by qRT-PCR (error bars, ±SEM).

DN1; $CD44^{+}CD25^{-}CD4^{-}CD8^{-}$, DN2; $CD44^{+}CD25^{+}CD4^{-}CD8^{-}$, DN3; $CD44^{-}CD25^{+}CD4^{-}CD8^{-}$, DN4; $CD44^{-}CD25^{-}CD4^{-}CD8^{-}$, and preDP $TCR\beta^{lo}CD69^{lo}CD4^{+}CD8^{+}$.


Twist2
mRNA expression
(normalized to β-actin)
0.0025
0.002
0.0015
0.001
0.0005
0
DN1
DN2
DN3
DN4
preDP

TCR signaling. Unlike the former feature is associated with pre-TCR signaling, the latter feature is induced by TCR and MHC interaction during preDP and postDP transition. To test whether TCR-dependent Twist2 induction is a transcriptional level, mRNA of *Twist2* was detected by quantitative realtime-PCR after reverse transcription (qRT-PCR). *Twist2* expression was higher in postDP thymocytes than preDP thymocytes (Figure 3).

To decipher the roles of Twist2 during DP to SP transition, *Twist2*-floxed mice were crossed with *Cd4*-Cre mice. *Twist2* was deleted at preDP stage in *Twist2*$^{f/f}$ x *Cd4*-Cre mice (hereafter, Twist2 cKO) (Figure 4). *Twist2* cKO mice displayed decreased thymic cellularity. However, the phenotype of early stages of thymocyte development, TCRβ^{lo}CD69^{lo} and TCRβ$^{int/hi}$CD69^{hi}, was comparable between *Twist2* cKO and control mice (Figure 5). However, the frequency of TCRβ^{hi}CD69^{lo} population was decreased in *Twis2* cKO mice compared with control mice. Consistent with the decreased percentage of TCRβ^{hi}CD69^{lo} population in *Twist2* cKO mice, the cell number of TCRβ^{hi}CD69^{lo} population was also decreased in *Twist2* cKO mice (Figure 6). However, in contrast to the normal frequency of TCRβ^{lo}CD69^{lo} population in *Twist2* cKO mice, cell number of this population was decreased in *Twist2* cKO mice. The cell types of TCRβ^{lo}CD69^{lo} population are heterogeneous including DN and preDP cells. Though the *Twist2* deletion was occurred at preDP stage and thus precluded the effect of *Twist2* deletion at DN stage, thymocyte development at DN stage of *Twist2* cKO mice was investigated by staining

with CD44 and CD25. As expected, *Twist2* cKO mice displayed normal phenotype at DN stage (Figure 7).

Twist2 regulates TCR sensitivity of thymocytes

TCRβ^{hi}CD69^{lo} population is regarded as a post-selected thymocytes and located at the medullary area of thymocytes (Fu and others, 2009). In the medulla of thymus, positively-selected thymocytes in the cortex of thymus are finally supervised by self-pMHCs on APCs. In particular, thymocytes that could response to TRAs presented on mTECs are eliminated in the medulla. To substrate the possibility that decreased TCRβ^{hi}CD69^{lo} population in *Twist2* cKO mice is due to the apoptosis, apoptotic cells were detected by staining with Annexin-V. Interestingly, apoptotic cells in TCRβ^{hi}CD69^{lo} population were increased in *Twist2* cKO mice compared with control mice (Figure 8). Moreover, apoptotic cells in TCRβ$^{int/hi}$CD69^{lo} population were also increased in *Twist2* cKO mice (Figure 8). Since these two populations shares the characteristics that receiving TCR signaling and sensitive to TCR stimulation, the absence of Twist2 might increase the TCR sensitivity of immature thymocytes. TCR sensitivity is determined by two factors, TCR and pMHC, respectively. However, each TCR exhibits unique sensitivity depending on the

Figure 3. *Twist2* expression is up-regulated in postDP thymocytes.

A. Sorting strategy of preDP and postDP population

B. *Twist2* expression was detected in both preDP and postDP cells by qRT-PCR. Expression level of *Twist2* is normalized to the results of preDP cells (error bars, ±SEM).

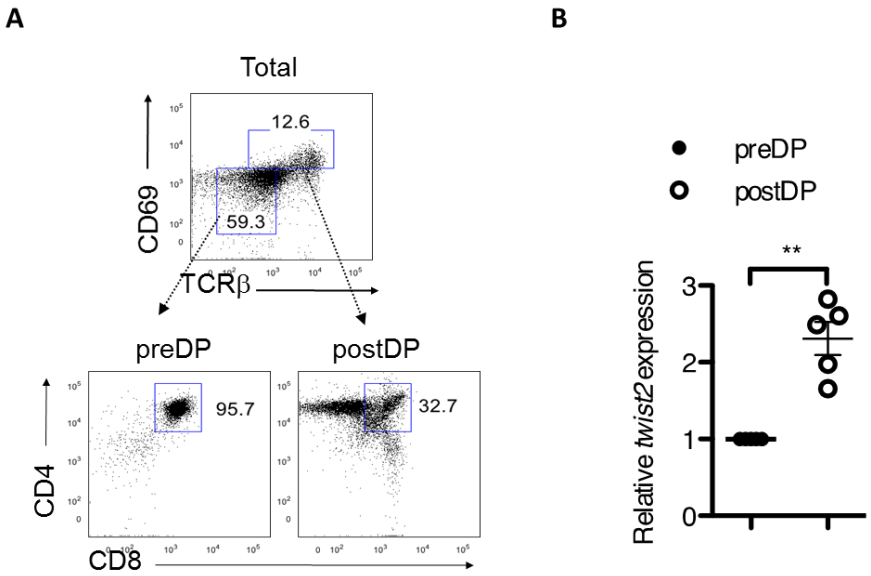
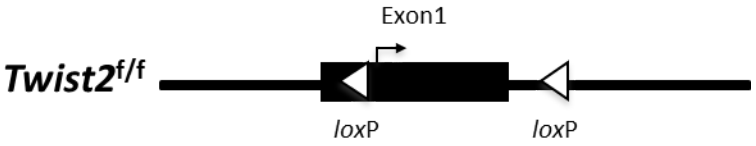

A
Total
12.6
59.3
CD69
TCRβ
preDP
postDP
95.7
32.7
CD4
CD8
B
preDP
postDP
**
Relative twist2 expression
3
2
1
0

Figure 4. *Twist2* expression pattern in *Twist2* cKO mice.

A. Schematic diagram of *Twist2*-floxed mice

B. Twist2 expression was detected by intracellular staining with anti-Twist2 monoclonal antibody. Relative expression of Twist2 (*Twist2* cKO/control) in each subsets is summarized (error bars, ±SEM).

A


Exon1
Twist2f/f
loxP
loxP

B

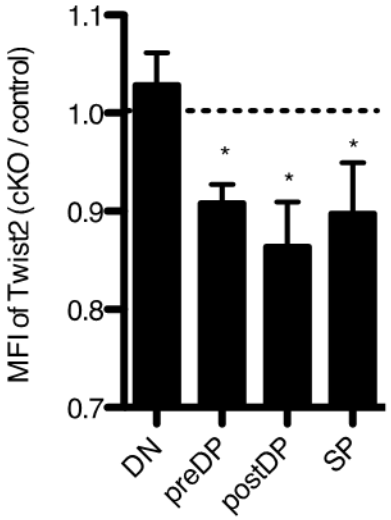

MFI of Twist2 (cKO / control)
1.1
1.0
0.9
0.8
0.7
*
*
*
DN
preDP
postDP
SP

Figure 5. Thymocyte profiles of *Twist2* cKO mice.

A. Total thymocytes of *Twist2* cKO and control mice (error bars, ±SEM)

B. CD4/CD4 plots of thymocytes in indicated mice. Numbers indicate the frequencies of each subsets.

C. TCRβ/CD69 plots of thymocytes in indicated mice. Numbers indicate the frequencies of each subsets.

D. The frequencies of each subsets are summarized (error bars, ±SEM).

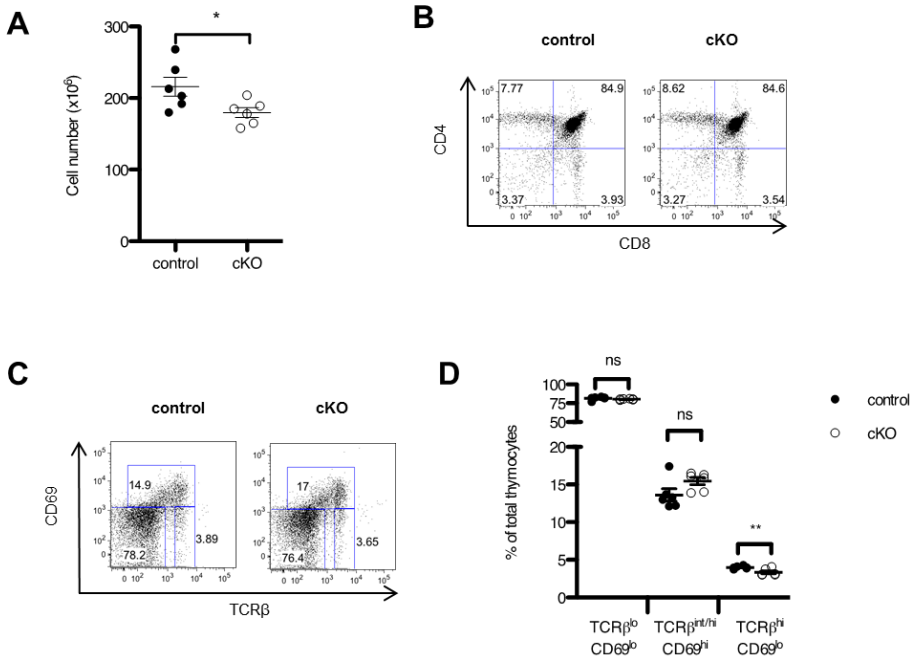

A
Cell number (x10^6)
300
200
100
0
control
cKO
*
B
control
cKO
CD4
CD8
7.77
84.9
3.37
3.93
8.62
84.6
3.27
3.54
C
control
cKO
CD69
TCRβ
14.9
78.2
3.89
17
76.4
3.65
D
% of total thymocytes
100
75
50
20
15
10
5
0
ns
ns
**
control
cKO
TCRβlo CD69lo
TCRβint/hi CD69hi
TCRβhi CD69lo

Figure 6. Cell number of TCRβ^{hi}CD69^{lo} population is decreased in *Twist2* cKO mice.

Cell numbers in each subsets are summarized (error bars, ±SEM)

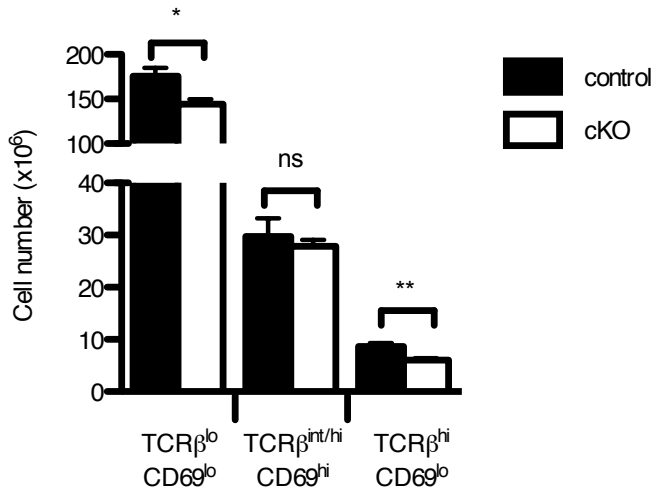

*
200
150
100
40
30
20
10
0
Cell number (x10^6)
ns
**
control
cKO
TCRβ^lo CD69^lo
TCRβ^int/hi CD69^hi
TCRβ^hi CD69^lo

Figure 7. The phenotype of DN stage in *Twist2* cKO mice.

A. CD44/CD25 plots gated on $CD4^{-}CD8^{-}$ population

B. Cell numbers of DN stage are summarized (error bars, ±SEM)

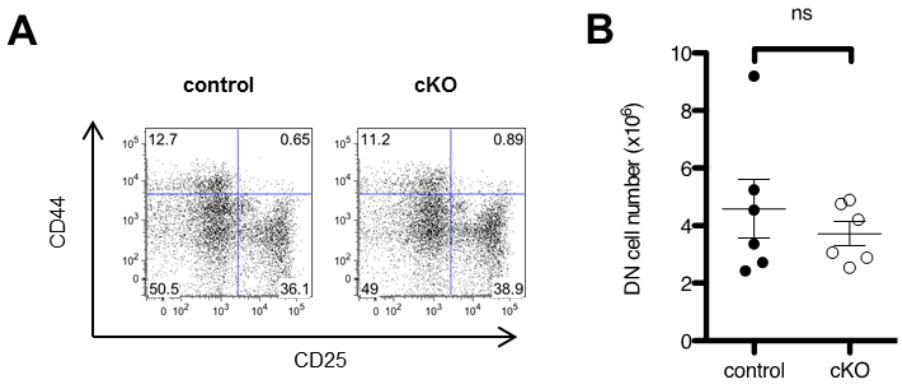
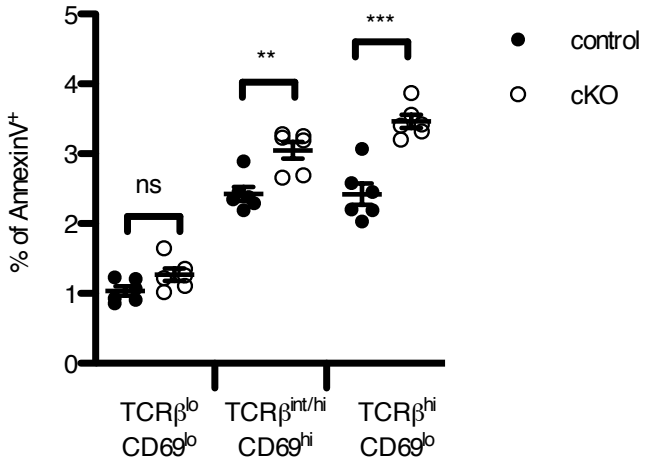

A
control
cKO
12.7
0.65
50.5
36.1
11.2
0.89
49
38.9
CD44
CD25
B
ns
DN cell number (x10^6)
control
cKO

Figure 8. Apoptosis in activated thymocytes are increased in *Twist2* cKO mice

Apoptotic cells were detected by staining with Annexin V kit (BD Biosciences following manufacturer's instruction. The percentage of Annexin V+ cells in each subsets are summarized (error bars, ±SEM).


5
4
3
2
1
0
% of AnnexinV+
ns
**
***
control
cKO
TCRβlo CD69lo
TCRβint/hi CD69hi
TCRβhi CD69lo

encountered pMHC. Since it is too complicated in polyclonal TCR condition, TCR transgenic mice system is employed to test TCR sensitivity. Thymocytes from TCR transgenic mice express only one kind of TCR on their surface. In addition, this system is better than polyclonal TCR condition because *in vitro* experiments are possible. Since the peptide is specifically identified for each TCR transgenic mice, both quality and quantity controls are possible in *in vitro* experiments. To generate *Twist2* cKO mice bearing monoclonal TCR, *Twist2* cKO mice were crossed with $Rag2^{-/-}$ x DO11.10 TCR transgenic mice (hereafter, DO-Tg). *Twist2* cKO x DO-Tg mice displayed normal thymocyte development (Figure 9). However, when thymocytes from *Twist2* cKO x DO-Tg mice were stimulated with $OVA_{323-339}$ peptides (OVA_p) presented on MHC-matching APCs, DP cell viability of Twist2 cKO x DO-Tg thymocytes was lower than that of control DO-Tg thymocytes (Figure 10). Consequently, these results suggest that Twist2 regulates the TCR sensitivity of thymocytes during thymocyte development.

Twist2 **expression is decreased in the negatively selected thymocytes**

The expression of Twist2 was higher in the postDP thymocytes than that in the preDP thymocytes. PostDP thymocytes include both positively-selected and negatively-selected thymocytes. To distinguish these two population and

Figure 9. Thymocyte profiles of Twist2 x DO11.10 TCR-Tg mice

A. CD4/CD8 and KJ1-26/CD24 profiles of thymocytes. Numbers indicate the frequencies of each subsets.

B Cell numbers of total thymocytes and mature DO11.10 TCR^{+} thymocytes ($KJ1\text{-}26^{hi}CD24^{lo}$) are summarized (error bars, ±SEM)

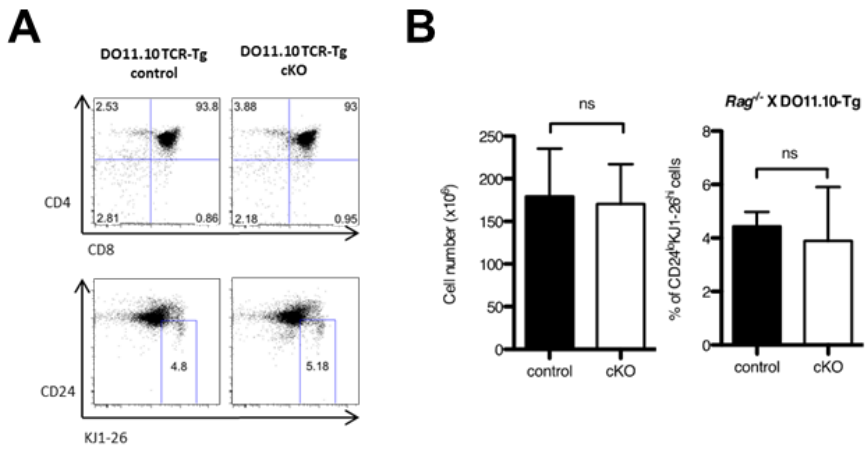
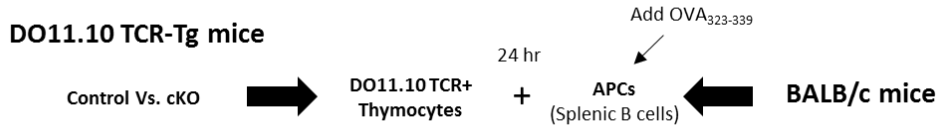

A
DO11.10TCR-Tg control
DO11.10TCR-Tg cKO
2.53
93.8
2.81
0.86
3.88
93
2.18
0.95
CD4
CD8
4.8
5.18
CD24
KJ1-26
B
ns
Cell number (x10⁶)
250
200
150
100
50
0
control
cKO
Rag-/- X DO11.10-Tg
ns
% of CD24hiKJ1-26hi cells
8
6
4
2
0
control
cKO

Figure 10. DP cell viability is decreased in *Twist2* cKO x DO11.10 TCR-Tg thymocytes.

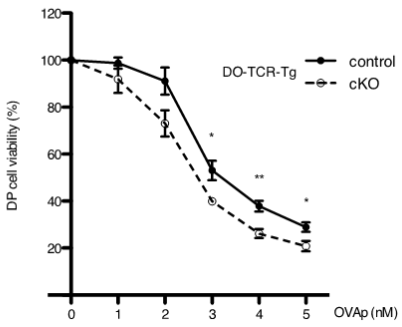
A. Schematic diagram to detect DP cell viability.

B. DO11.10 TCR^+ thymocytes from *Twist2* cKO and control mice were co-cultured with OVAp loaded splenic B cells of MHC-matched BALB/C mice for 24 hr. Indicated concentrations of OVAp were pre-treated to B cells 30 min prior to co-culture. DP cell viabilities were detected by the frequency of DP thymocytes and normalized the DP percentage of non-treated cells (error bars, ±SEM).

A


DO11.10 TCR-Tg mice
Control Vs. cKO
DO11.10 TCR+
Thymocytes
24 hr
Add OVA323-339
+
APCs
(Splenic B cells)
BALB/c mice

B


DP cell viability (%)
120
100
80
60
40
20
0
1
2
3
4
5
OVAp (nM)
DO-TCR-Tg
control
cKO
*
**
*

determine whether the expression of *Twist2* is differentially regulated depending on these two populations, thymocytes from *TCRα*$^{-/-}$ mice were activated with either weak (PMA 0.1 ng/ml and ionomycin 0.2μM, mimicking positive selection TCR signal-strength; P0.1/I200) or strong (PMA 10 ng/ml and ionomycin 0.5 μM, mimicking negative selection TCR signal-strength; P10/I500) (Shao and others, 1999; Wilkinson and Kaye, 2001). As expected, *Twist2* expression was up-regulated in *TCRα*$^{-/-}$ thymocytes after stimulation with weak condition (Figure 11).

Interestingly, the stimulation with a strong condition repressed *Twist2* expression in *TCRα*$^{-/-}$ thymocytes (Figure 11). To clarify whether, negative selection-strength of TCR signal represses *Twist2* expression, DO-Tg mice were injected OVA_p by intraperitoneal injection (Figure 12A). Without OVA_p, most DO11.10 TCR^+ thymocytes undergo positive selection, while OVA_p injection makes DO11.10 TCR^+ thymocytes undergo negative selection (Fassett and others, 2012). Consistent with down-regulation of *Twist2* expression after P10/I500 stimulation in *TCRα*$^{-/-}$ thymocytes, *Twist2* expression in negatively-selected DO11.10 TCR^+ thymocytes by OVA_p injection was lower than that in positively-selected DO11.10 TCR^+ thymocytes (Figure 12B).

Figure 11. *Twist2* expression pattern depending on TCR signal-strength.

Thymocytes from *TCRα*$^{-/-}$ mice were activated with either weak (P0.1/I200) or strong (P10/I500) conditions. *Twist2* expression was detected by qRT-PCR (error bars, ±SEM).

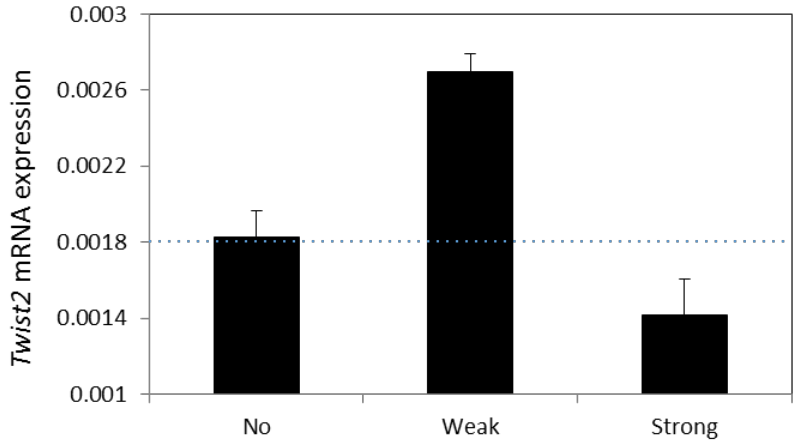
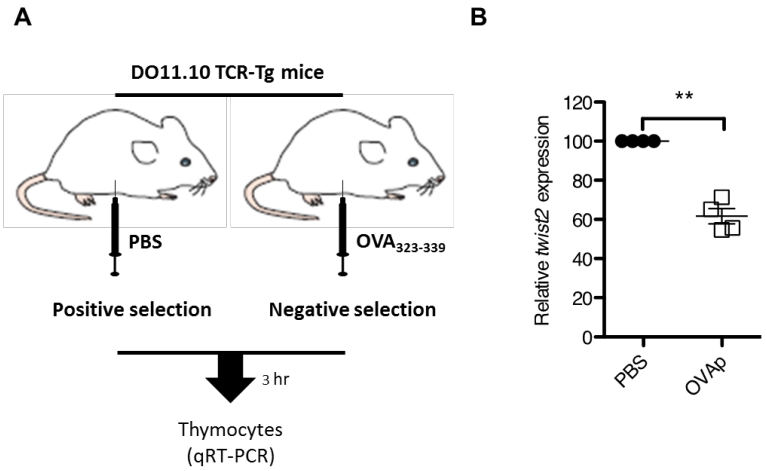

0.003
0.0026
0.0022
0.0018
0.0014
0.001
Twist2 mRNA expression
No
Weak
Strong

Figure 12. *Twist2* expression is down-regulated in negatively-selected thymocytes.

A. Schematic diagram of the process to obtain positively-selected and negatively-selected thymocytes.

B. DO-Tg mice were intraperitoneally injected with either PBS or OVAp. After 3 hr, *Twist2* expression was detected in each thymocytes by qRT-PCR. Relative *Twist2* expression to the results of thymocytes from PBS-injected mice were summarized (error bars, ±SEM).


A
DO11.10 TCR-Tg mice
PBS
OVA323-339
Positive selection
Negative selection
3 hr
Thymocytes
(qRT-PCR)
B
Relative twist2 expression
120
100
80
60
40
20
0
**
PBS
OVAp

Twist2 regulates activation-induced cell death of thymocytes

Twist2 expression was differentially regulated in two PMA/IM cocktails, P0.1/I200 and P10/I500, respectively. In particular, *Twist2* expression was down-regulated by P10/I500 condition, which causes apoptosis of thymocytes (Figure 13). In addition, DP cell viability was decreased in the absence of Twist2 after OVA_p stimulation. Thus, it is possible that Twist2 regulates TCR-mediated AICD of thymocytes.

To substrate this possibility, *Twist2* cKO mice were crossed with *TCRα*$^{-/-}$ mice to obtain TCR-unexperienced thymocytes and then stimulated with PMA and ionomycin (PMA/IM). Consistent with the previous data showing increased TCR sensitivity in *Twist2* cKO thymocytes, PMA/IM-mediated AICD was increased in *TCRα*$^{-/-}$ x *Twist2* cKO thymocytes compared with control mice (Figure 14A). Surprisingly, in contrast to the data from *Twist2* cKO mice, PMA/IM-mediated AICD was dramatically decreased in the thymocytes from *TCRα*$^{-/-}$ x *Twist2*-Tg mice compared with that in NLC mice (Figure 14B). To clarify whether Twist2 specifically represses TCR-mediated AICD of thymocytes, thymocyte apoptosis was detected after the treatment with dexamethasone, which induces TCR-independent AICD of thymocytes. Unlike PMA/IM-mediated AICD of thymocytes, Twist2 failed to suppress Dex-mediated AICD of thymocytes (Figure 14B). These data suggest that Twist2 specifically regulates TCR-mediated AICD of thymocytes.

Twist2 impairs negative selection of thymocytes

Self-reactive thymocytes are eliminated by negative selection in the thymus. Self-reactivity is determined by the TCR and self-pMHC interaction and the resulting TCR signal-strength. Since Twist2 repressed TCR-mediated AICD of thymocytes, it is likely to test whether Twist2 regulates negative selection of thymocytes. To validate the roles of Twist2 in negative selection, OT-II TCR x RIP-mOVA-Tg mice system is employed. As previously mentioned, most thymocytes in TCR transgenic mice undergo positive selection. Like DO11.10 TCR^+ thymocytes, OT-II TCR^+ thymocytes also recognize OVA_p and undergo apoptosis. RIP-mOVA transgenic mice express ovalbumin under rat insulin promoter. It has been reported that ovalbumin is expressed in the pancreas, kidney, and thymic medulla. Thus, in OT-II TCR x RIP-mOVA-Tg mice system, OT-II TCR^+ thymocytes encounter with OVA_p in the medullary area of thymus and undergo negative selection (Fassett and others, 2012).

To substrate the roles of Twist2 in negative selection, *Twist2*-Tg mice were crossed with OT-II TCR and RIP-mOVA-Tg mice. In this system, OT-II TCR-Tg and OT-II TCR x *Twist2*-Tg mice undergo positive selection, while OT-II TCR x RIP-mOVA-Tg and OT-II TCR x RIP- mOVA x *Twist2*-Tg mice

Figure 13. PMA/IM conditions mimicking positive and negative selection

Thymocytes from *TCRα*-/- mice were activated with non, P0.1/I200 (PMA 0.1 ng/ml and ionomycin 0.2μM), and P10/I500 (PMA 10 ng/ml and ionomycin 0.5 μM). Apoptotic cells were detected after staining with AnnexinV following manufacturer's instruction. The numbers indicate the percentage of AnnexinV$^+$ cells.

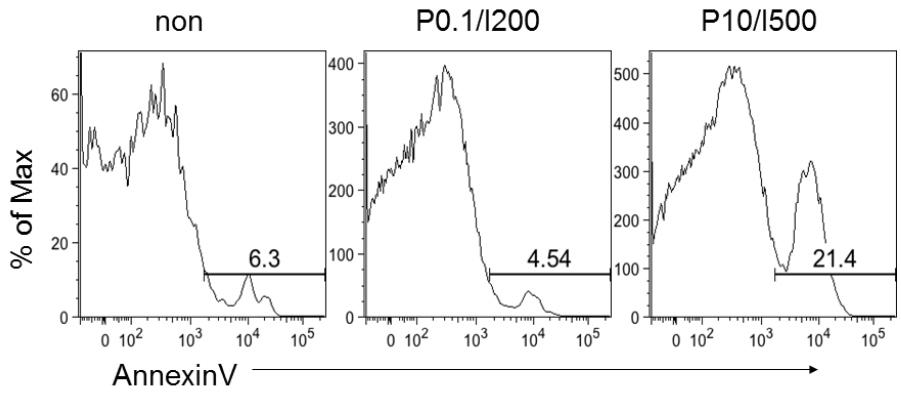

non
P0.1/I200
P10/I500
% of Max
6.3
4.54
21.4
AnnexinV

Figure 14. Twist2 regulates TCR-mediated activation-induced cell death of thymocytes.

A and B. Thymocytes from indicated mice were activated with either non or PMA/IM (PMA 10 ng/ml and ionomycin 0.5 μM). Apoptotic cells were detected by staining with AnnexinV following manufacturer's instruction (error bars, ±SEM).

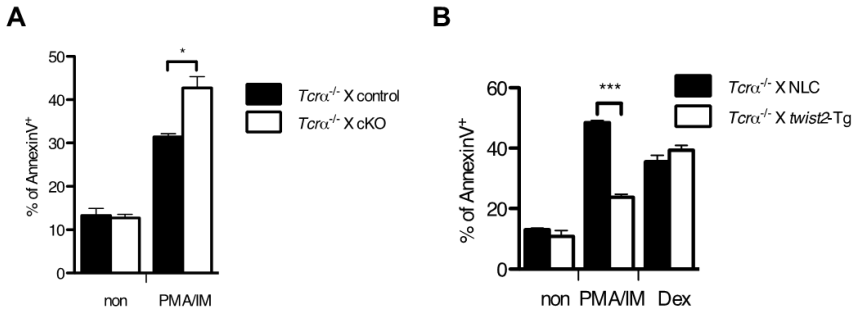
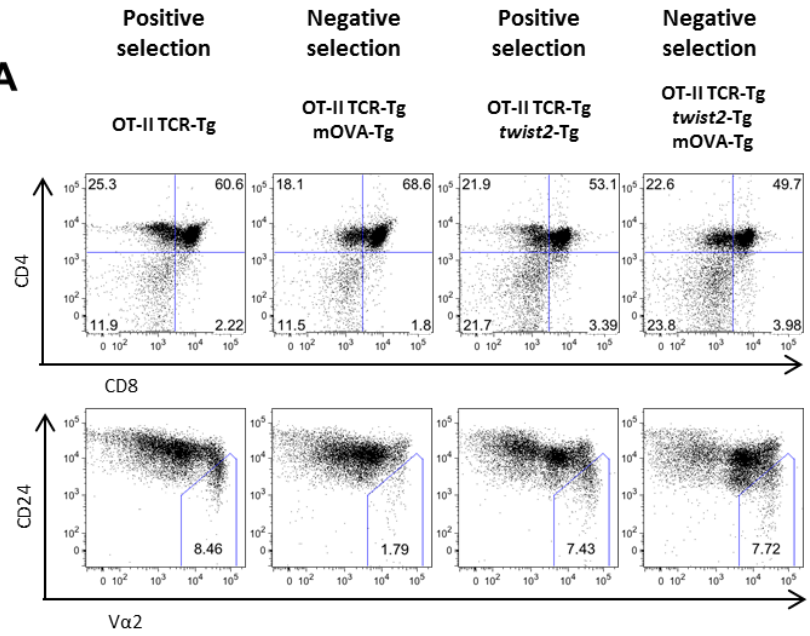

A
B
% of AnnexinV+
50
40
30
20
10
0
non
PMA/IM
*
Tcrα-/- X control
Tcrα-/- X cKO
60
40
20
0
Dex
***
Tcrα-/- X NLC
Tcrα-/- X twist2-Tg

Figure 15. Negative selection is impaired in *Twist2*-Tg mice.

A. CD4/CD8 and CD24/Vα2 plots of four different OT-II TCR-Tg lines (±RIP-mOVA-Tg and ±*Twist2*-Tg). Numbers indicate the frequencies of each subsets.

B. The frequency of mature OT-II TCR^{+} thymocytes indicated mice were summarized (error bars, ±SEM).

A



- **Vα2:** OT-II TCR β chain
- **CD24^{lo}Vα2^{hi} population**: mature OT-II TCR+ thymocytes

B

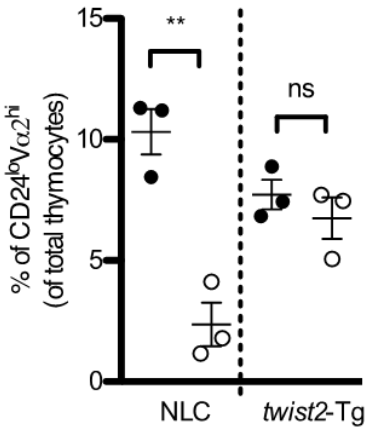
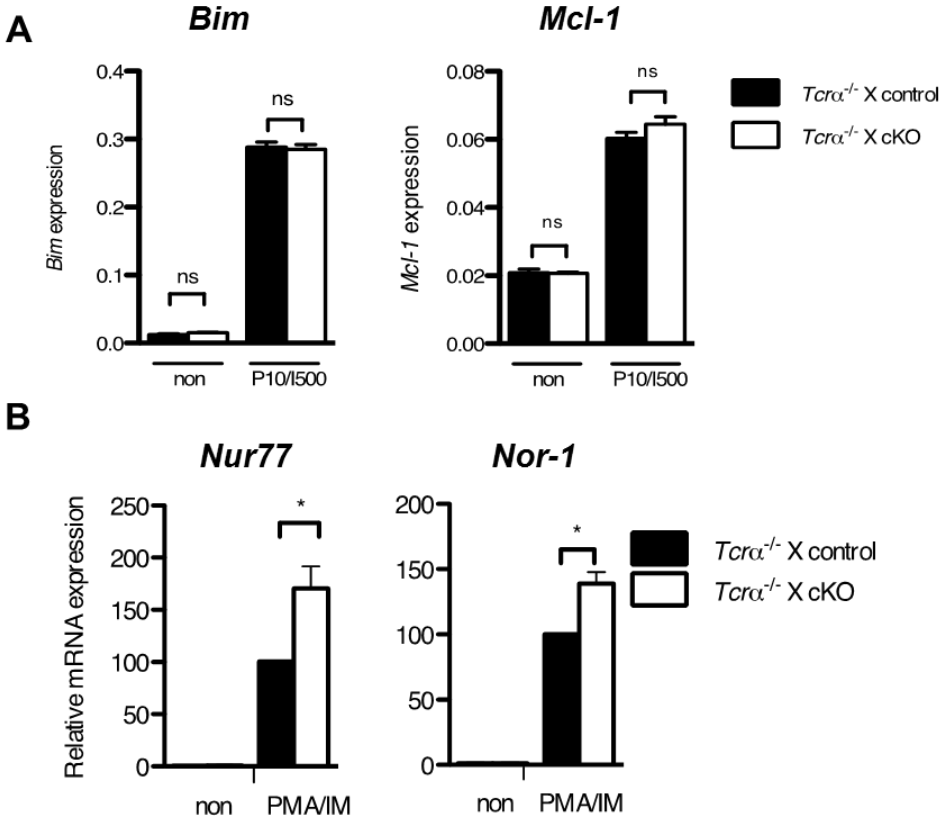


Figure 16. *Nur77* and *Nor-1* expression is increased in the absence of *Twist2* after TCR stimulation.

A and B. Thymocytes from Twist2 cKO and control mice under *TCRα*$^{-/-}$ background were activated with PMA/IM. Indicated gene expression was detected by qRT-PCR (error bars, ±SEM).


A
Bim
Mcl-1
Bim expression
Mcl-1 expression
0.4
0.3
0.2
0.1
0.0
0.08
0.06
0.04
0.02
0.00
ns
non
P10/I500
Tcrα-/- X control
Tcrα-/- X cKO
B
Nur77
Nor-1
Relative mRNA expression
250
200
150
100
50
0
*
non
PMA/IM

undergo negative selection (Figure 15A). Mature OT-II TCR^+ thymocytes ($CD24^{lo}V\alpha2^{hi}$) were dramatically decreased in OT-II TCR x RIP-mOVA-Tg mice compared with OT-II TCR mice (Figure 15A and B). However, this reduction induced by negative selection of mature OT-II TCR^+ thymocytes was not detected in *Twist2*-Tg mice (OT-II TCR x *Twist2*-Tg Vs. OT-II TCR x RIP-mOVA x *Twist2*-Tg) (Figure 15A and B).

Twist2 regulates *Nur77* and *Nor-1* expression in activated thymocytes

Inhibition of thymocyte AICD can be explained by two different approaches, promoting pro-survival molecules and suppressing pro-apoptotic molecules. Among them, *Nur77* and *Nor-1* expressions were significantly increased in the absence of *Twist2* after TCR stimulation, while *Mcl-1* (pro-survival molecule) and *Bim* (pro-apoptotic molecule) expressions were comparable (Figure 16).

It is interesting because *Nur77*-null mice displayed abnormal negative selection in OT-II TCR x RIP-mOVA-Tg mice (Fassett and others, 2012). To test whether impaired negative selection in *Twist2*-Tg mice under OT-II TCR x RIP-mOVA-Tg system, Nur77 expression was detected by intracellular staining with anti-Nur77 antibody. The frequency of $Nur77^{hi}$ cells and the expression level (MFI) of Nur77 were increased in negatively-selected mature

OT-II TCR^+ cells compared with positively-selected mature OT-II TCR^+ cells (Figure 17). In *Twist2*-Tg mice, however, the percentage of $Nur77^{hi}$ cells and the MFI of Nur77 were decreased compared with negatively-selected mature OT-II TCR^+ cells though Nur77 expression was still slightly, but significantly increased in negatively-selected mature OT-II TCR^+ thymocytes of *Twist2*-Tg mice compared with positively-selected mature OT-II TCR^+ thymocytes of NLC mice (Figure 17).

TCR signaling is not affected by Twist2

It has been reported that *Nur77* expression pattern is positively correlated with TCR signal-strength (Fassett and others, 2012). Thus, it is possible that decreased *Nur77* expression in *Twist2*-Tg mice is due to the attenuated TCR signaling. To rule out this possibility, TCR signaling is checked using three independent methods, phosphotyrosine status, Ca^{2+} influx, and CD69 induction after TCR stimulation. First two methods are for the testing of proximal TCR signaling and the last one is for the checking of distal TCR signaling. After TCRs encounters with pMHCs, activated CD3 complex phosphorylates adaptor molecules Lck and Zap70. Then, these phosphorylated adaptor molecules activate PLCγ and LAT. Activated PLCγ induces Ca^{2+} influx and activates PKC pathway. LAT activates RAS, and activated RAS

Figure 17. Nur77 expression is decreased in negatively-selected thymocytes of *Twist2*-Tg mice.

A. Histogram of intracellular Nur77 protein of $CD24^{lo}V\alpha2^{hi}$ population in indicated mice. Numbers indicate the frequency of $Nur77^{hi}$ cells. Tinted; Non-RIP-mOVA-Tg background, Line; RIP-mOVA-Tg background.

B. The frequencies of $Nur77^{hi}$ cells in $CD24^{lo}V\alpha2^{hi}$ population were summarized (error bars, ±SEM).

C. MFI of Nur77 of $CD24^{lo}V\alpha2^{hi}$ population in indicated mice were summarized (error bars, ±SEM).

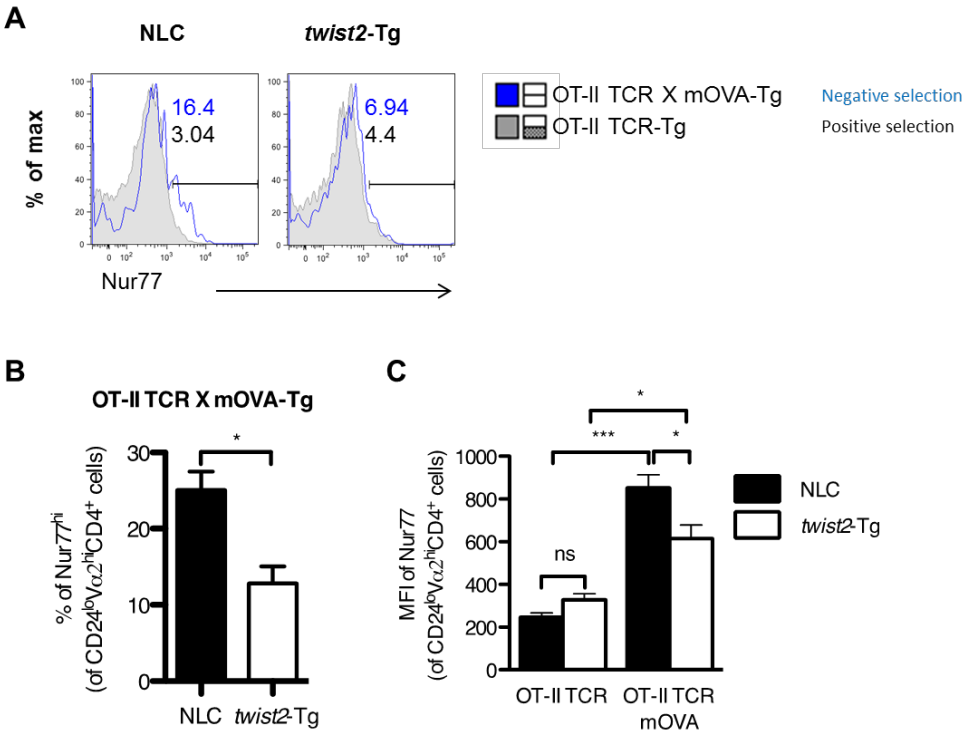
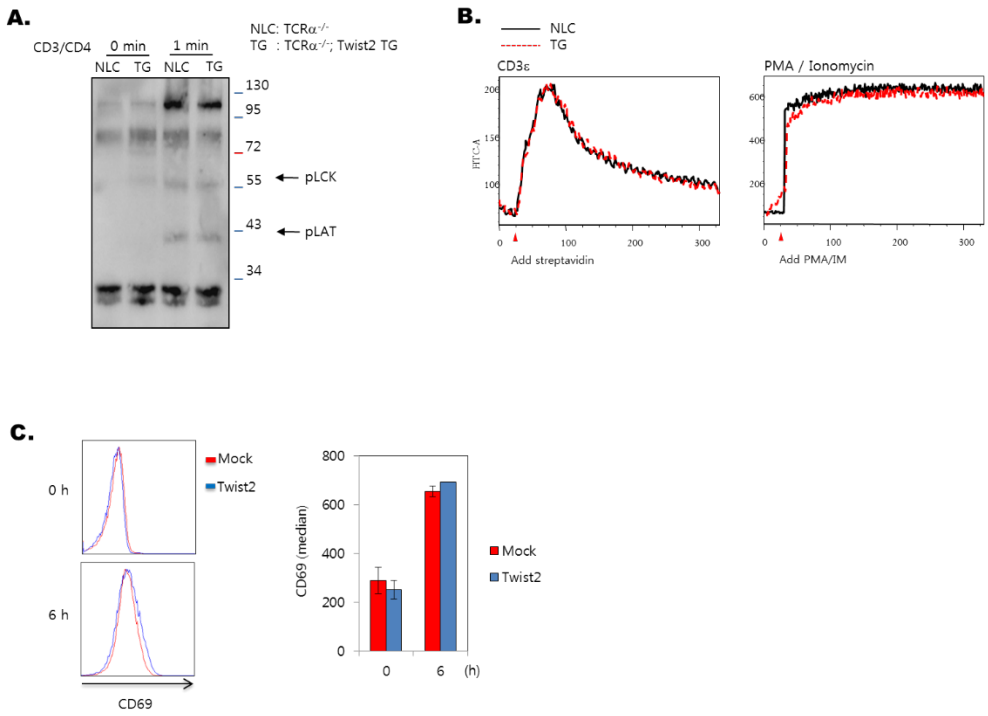

A
NLC
twist2-Tg
% of max
16.4
3.04
6.94
4.4
Nur77
OT-II TCR X mOVA-Tg
OT-II TCR-Tg
Negative selection
Positive selection
B
OT-II TCR X mOVA-Tg
% of Nur77hi (of CD24loVα2hiCD4+ cells)
NLC
twist2-Tg
C
MFI of Nur77 (of CD24loVα2hiCD4+ cells)
ns
OT-II TCR
OT-II TCR mOVA
NLC
twist2-Tg

Figure 18. TCR signaling is not affected by Twist2.

A. Thymocytes from indicated mice were stained with anti-CD3-biotin and anti-CD4-biotin antibodies. After incubation with anti-streptavidin antibody for 1 min, whole cell lysates were prepared. Phosphotyrosine status in whole cell lysates were detected using 1:1 mixture of p-Tyr (PY20) and p-Tyr (PY99) antibodies.

B. Thymocytes of indicated mice were stained with anti-CD3ε-biotin antibody. The added time of anti-streptavidin antibody or PMA/IM was indicated as arrowhead. Ca^{2+} influx was detected by FACS Canto II using Fluo-4 NW calcium assay kit (starter) (Molecular Probes) following manufacturer's instruction.

C. 16610D9 cells were transfected with either Twist2 expression or mock vector. After 48 hr, cells were further activated for 6 hr with PMA/IM. CD69 expression was detected by FACS Canto II using anti-CD69 antibody. Median value of CD69 expression were summarized (error bars, ±SEM)


A.
CD3/CD4
0 min
1 min
NLC TG NLC TG
NLC: TCRα-/-
TG : TCRα-/-; Twist2 TG
130
95
72
55
pLCK
43
pLAT
34
B.
NLC
TG
CD3ε
PMA / Ionomycin
FITC-A
Add streptavidin
Add PMA/IM
C.
0 h
6 h
Mock
Twist2
CD69
CD69 (median)
800
600
400
200
0
0
6
(h)
Mock
Twist2

triggers ERK and/or JNK pathway. CD69 is a widely used marker indicating T cell activation.

When thymocytes from *Twist2*-Tg and NLC mice under *TCRα*$^{-/-}$ mice were activated with anti-CD4 and anti-CD3 antibodies, phosphorylation of LCK and LAT were comparable between *Twist2*-Tg and NLC thymocytes (Figure 18A). Ca^{2+} influx was tested by stimulation with either anti-CD3 antibody or PMA/IM treatment. In both cases, Twist2 did not alter the Ca^{2+} influx (Figure 18B). CD69 induction was tested in 16610D9 thymoma cells after transfection with Twist2 expression vector and activated with PMA/IM. CD69 was normally induced in Twist2-transfected 16610D9 cells after PMA/IM treatment (Figure 18C). Collectively, these data suggest that decreased *Nur77* expression in the thymocytes of *Twist2*-Tg mice is not due to the attenuated TCR signaling by Twist2 over-expression.

Twist2 directly binds to the promoters of *Nur77* and *Nor-1*

Since Twist2 is a general transcription factor and represses *Nur77* and *Nor-1* expression in activated thymocytes, the binding motifs of Twist2 on each *Nur77* and *Nor-1* promoter was investigated. In *Nur77* promoter, the MEF2 sites has been well established as a key element in the TCR-dependent regulation of *Nur77* expression (Liu and others, 1994; Woronicz and others, 1995; Youn and others, 2000; Youn and Liu, 2000; Youn and others, 1999).

Unlike *Nur77* promoter, there are few information about the *Nor-1* promoter. Only HDAC7 binding site (hereafter, HDAC7 site) on the *Nor-1* promoter was reported (Figure 19A) (Chen and others, 2009). To ascribe whether Twist2 binds to these motifs in each promoters, ChIP assay was conducted and Twist2 binding was detected by semi-qPCR using primer pairs including the MEF2 site and the HDAC7 site. As expected. Twist2 could bind to the MEF2 site and the HDAC7 site (Figure 19B).

Twist2 represses the promoter activities of *Nur77* and *Nor-1* via the MEF2 site and the HDAC7 site, respectively.

To assess whether Twist2 regulates *Nur77* expression via the binding to the MEF2 site of *Nur77* promoter, *Nur77* promoter including the MEF2 site were cloned and the promoter activity was detected in the presence of Twist2. Additionally, two *Nur77* promoters, mutation at the sequence of MEF2 site (named *Nur77*-mutMEF2) or containing only the sequence of MEF2 site (named *Nur77*-MEF2only), were also cloned to define whether Twist2 regulates *Nur77* expression via the MEF2 site (Figure 20A).

Consistent with the repression of *Nur77* expression, Twist2 inhibited the *Nur77* promoter activity. Interestingly, when the MEF2 site of *Nur77* promoter was mutated, Twist2 could not repress the *Nur77*-mutMEF2 promoter activity (Figure 20B). To validate whether repressive function of Twist2 on the *Nur77*

promoter is via the MEF2 site, the promoter activity of *Nur77*-MEF2only construct was detected in the presence of Twist2. The promoter activity of this construct was repressed by Twist2 in a dose dependent manner (Figure 20C). Taken together, these data suggest that Twist2 directly represses *Nur77* expression by binding to the MEF2 site of *Nur77* promoter.

Next, the promoter activities of various *Nor-1* promoter constructs were investigated in the presence of Twist2 (Figure 21A). Among the *Nor-1* promoter constructs (*Nor-1*, *Nor-1*-ΔHDAC7, and *Nor-1*-mutHDAC7), Twist2 only repressed intact *Nor-1* promoter construct (Figure 21B). Combining with the results showing Twist2 binds to the HDAC7 site of *Nor-1* promoter, these data suggest that Twist2 represses *Nor-1* expression via the HDAC7 site

Twist2 interacts with MEF2D and HDAC7 via C-terminal domain.

It has been reported that HDAC7 represses *Nur77* expression by interaction with MEF2D. MEF2D constitutively binds to the MEF2 site of *Nur77* promoter and serves as an anchor protein for other transcription factors. Twist2 also repressed *Nur77* expression by binding to the MEF2 site of *Nur77* promoter. Besides, it was reported that Twist2 interacts with MEF2C, which shares highly conserved sequence with MEF2D, and HDACs though the interaction between Twist2 and HDAC7 has not been reported (Gong and Li,

2002).

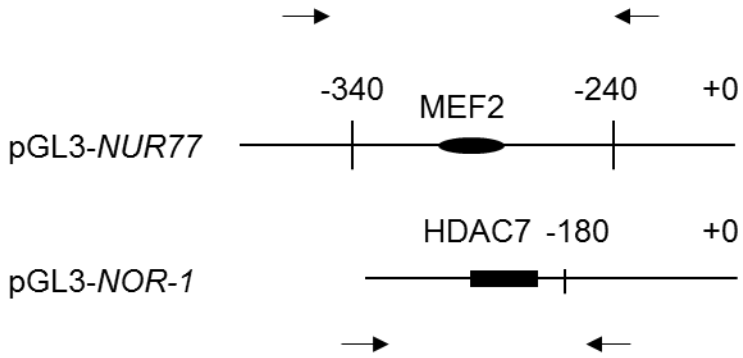
To test whether Twist2 interacts with MEF2D and HDAC7, bimolecular fluorescence complementation (BiFC) assay was employed. Using this assay, protein-protein interaction is easily detected in live cells using fluorescence microscope and FACS machine without any disruptions (Song and others, 2014; Sung and Huh, 2007). Briefly, two segmented subunits of VENUS protein, VN173 and VC155, respectively, are linked with interested proteins and then transfected into cells. If two interested proteins are proximate or interacted with each other, linked VENUS subunits are also combined as an intact VENUS protein. VENUS proteins are able to detect as a green fluorescence. To ascertain the possibility of the interaction between Twist2 and MEF2D or HDAC7, several VN/VC vectors inserted with Twist2, MEF2D, and HDAC7 were cloned. Additionally, C-terminal deleted form of Twist2 (hereafter, Twist2ΔC) was cloned into VC vector because C-terminal domain of Twist2 is required for the interaction between Twist2 and MEF2D. Thus, if Twist2 was able to interact with MEF2D, C-terminal domain of Twist2 might be required for Twist2/MEF2D interaction, too. When the interactions between Twist2 and MEF2D or Twist2ΔC and MEF2 were investigated using BiFC assay, only intact Twist2, not Twist2ΔC was able to interact with MEF2D (Figure 22). HDAC7 was also interacted with intact Twist2 protein, but not with Twist2ΔC (Figure 22). Since these three proteins binds to the MEF2 site of Nur77 promoter, these data imply the possibility of co-operation

Figure 19. Twist2 binds to the promoter of *Nur77* and *Nor-1* via the MEF2 site and the HDAC7 site, respectively.

A. Schematic diagram of the cis-elements on the *Nur77* and *Nor-1* promoter. Primer pairs used in ChIP assay was indicated as arrow.

B. ChIP assay was performed in thymocytes from DO11.10-TCR x *Twist2*-Tg mice. The binding of Twist2 on the *Nur77* promoter and the *Nor-1* promoter was detected by semi-qPCR and quantified using ImageJ software (error bars, ±SEM).

A


-340
MEF2
-240
+0
pGL3-*NUR77*
HDAC7
-180
+0
pGL3-*NOR-1*

B

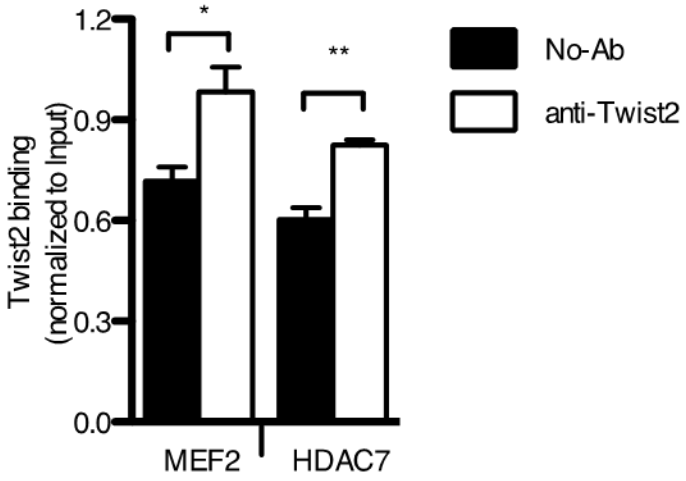

1.2
0.9
0.6
0.3
0.0
Twist2 binding
(normalized to Input)
*
**
No-Ab
anti-Twist2
MEF2
HDAC7

Figure 20. Twist2 represses the *Nur77* promoter activity via the MEF2 site.

A. Schematic diagram of various *Nur77* promoter constructs. Mutated sequence were indicated as underline in the sequence.

B and C. 16610D9 cells were transfected with indicated combination of promoters. At 48 hr, transfected cells were further activated with PMA/IM. Relative luciferase activity to mock transfected cells were summarized (error bars, ±SEM).

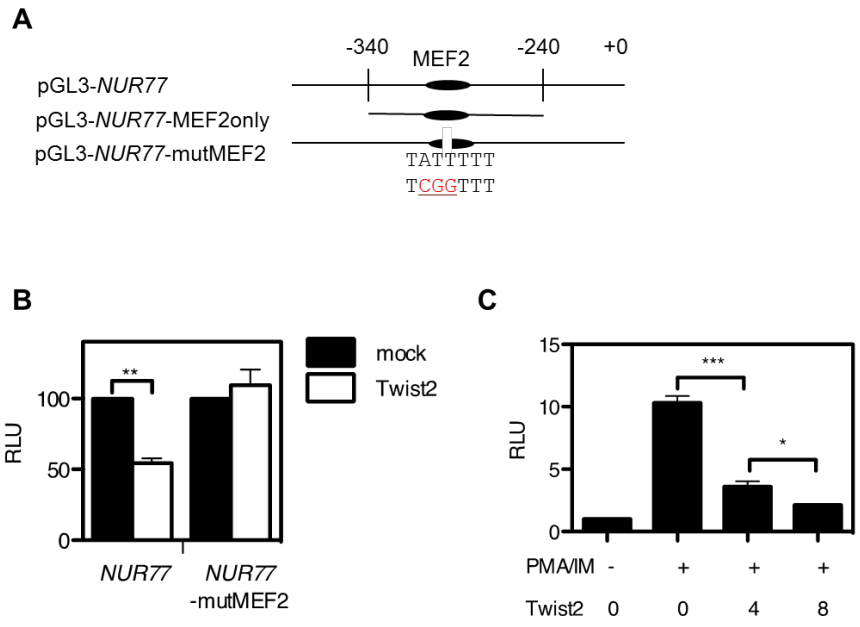
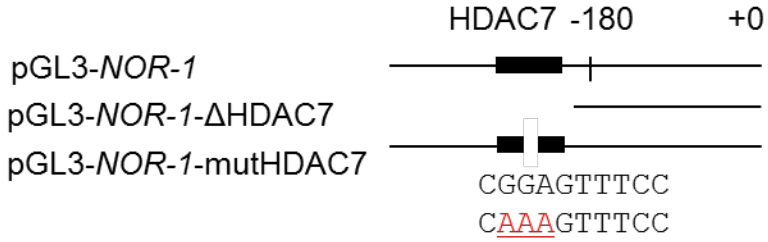

A
-340
MEF2
-240
+0
pGL3-NUR77
pGL3-NUR77-MEF2only
pGL3-NUR77-mutMEF2
TATTTTT
TCGGTTT
B
mock
Twist2
RLU
100
50
0
**
NUR77
NUR77
-mutMEF2
C
RLU
15
10
5
0
***
*
PMA/IM - + + +
Twist2 0 0 4 8

Figure 21. Twist2 represses the *Nor-1* promoter activity via the HDAC7 site.

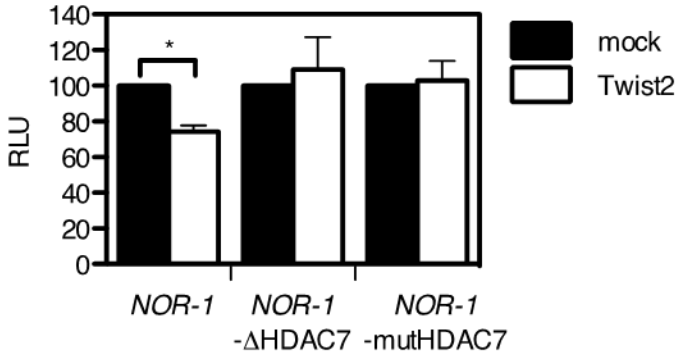
A. Schematic diagram of various *Nor-1* promoter constructs. Mutated sequences were indicated as underline in the sequence.

B and C. 16610D9 cells were transfected with indicated combination of promoters. At 48 hr, transfected cells were further activated with PMA/IM. Relative luciferase activity to mock transfected cells were summarized (error bars, ±SEM).

A


HDAC7 -180
+0
pGL3-*NOR-1*
pGL3-*NOR-1*-ΔHDAC7
pGL3-*NOR-1*-mutHDAC7
CGGAGTTTCC
CAAAGTTTCC

B


140
120
100
80
60
40
20
0
RLU
*
mock
Twist2
NOR-1
NOR-1 -ΔHDAC7
NOR-1 -mutHDAC7

between these proteins in *Nur77* regulation.

Twist2 interacts with MEF2D and HDAC7 in positively-selected thymocytes

The interaction of Twist2 and HDAC7 was confirmed using co-immunoprecipitation assay (Figure 23A). Consistent with the results from BiFC assay, Twist2ΔC did not interact with HDAC7 (Figure 23A). Since Twist2 expression is up-regulated in activated thymocytes, especially positively-selected thymocytes, and Twist2 might repress the *Nur77* expression in the thymocytes undergoing positive selection, Twist2 could interact with MEF2D and HDAC7 in positively-selected thymocytes. To clarify this issue, DP thymocytes from *Twist2*-Tg under DO-Tg background were sorted and physical interaction between Twist2, MEF2D, and HDAC7 were detected after immunoprecipitation using anti-Twist2 antibody. Consistent with the results using cell lines, Twist2 interacted with MEF2D and HDAC7 in positively-selected DP thymocytes (Figure 23).

C-terminal domain of Twist2 is necessary for the repression of the Nur77 promoter activity

Above data suggest that Twist2 represses *Nur77* expression via the interaction with MEF2D and HDAC7 and C-terminal domain of Twist2 is

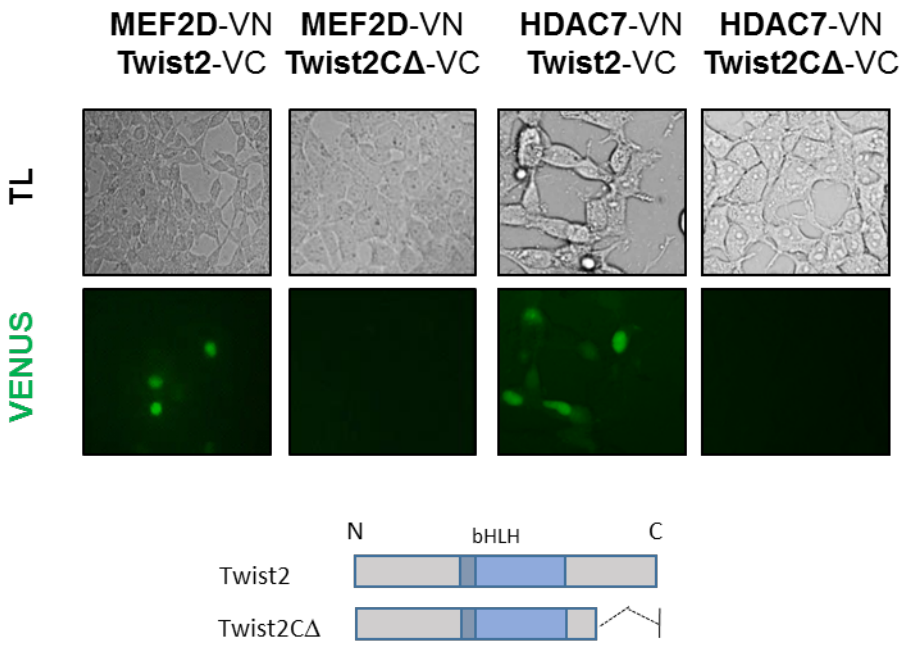
important for these protein-protein interactions. To demonstrate this model, the activity of *Nur77* promoter was measured after transfection with either Twist2 or Twist2ΔC. Consistent with previous data, Twist2 successfully repressed the *Nur77* promoter activity after PMA/IM stimulation (Figure 24). However, Twist2ΔC failed to repress the *Nur77* promoter (Figure 24).

C-terminal domain of Twist2 is required for the suppression of thymocyte AICD after TCR stimulation

It was revealed that C-terminal domain of Twist2 is crucial for the repression of the *Nur77* promoter activity. Subsequently, the effect of C-terminus-deletion of Twist2 was investigated in activated cells. 16610D9 cells were transfected with either Twist2 or Twist2ΔC and activated with PMA/IM after 48 hr. Unlike Twist2, Twist2ΔC could not suppress the AICD of thymocytes after PMA/IM stimulation (Figure 25). Taken together, these data suggest that Twist2 interacts with MEF2D and HDAC7 in positively selected thymocytes via the C-terminal domain and suppresses AICD of thymocytes by repressing *Nur77* expression. On the contrary, in negative selection, Twist2 expression is down-regulated and thus *Nur77* is highly expressed to cause AICD of thymocytes.

Figure 22. Twist2 interacts with MEF2D and HDAC7 via C-terminal domain

Indicated combinations of VN/VC vectors were transfected into 293T cells. After 48 hr, VENUS fluorescence were detected using fluorescence microscope. Schematic diagrams of Twist2 and Twist2ΔC were drawn.

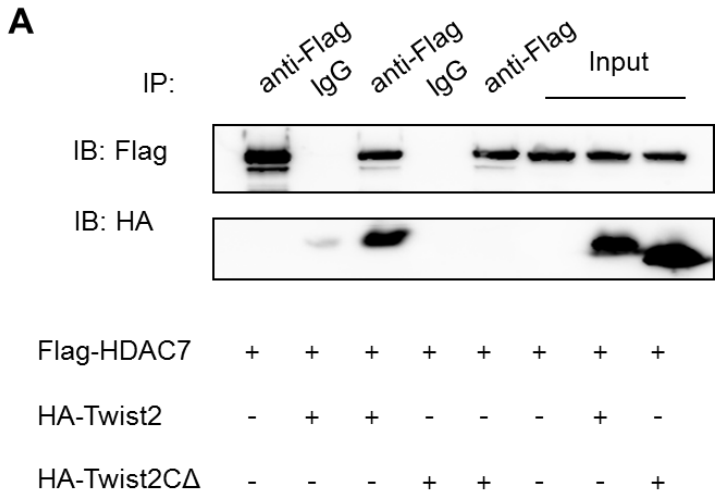

MEF2D-VN
Twist2-VC
MEF2D-VN
Twist2CΔ-VC
HDAC7-VN
Twist2-VC
HDAC7-VN
Twist2CΔ-VC
TL
VENUS

N
bHLH
C
Twist2
Twist2CΔ

Figure 23. Twist2 interacts with MEF2D and HDAC7 in positively-selected thymocytes.

A. 293T cells were transfected with indicated combinations of expression vectors. Cell lysates were immunoprecipitated by using and Flag antibody. Immunoprecipitated HDAC7, Twist2, and Twist2ΔC were detected by western blotting.

B. Cell lysates of sorted DP thymocytes obtained from Twist2 x DO-Tg mice were immunoprecipitated using anti-Twist2 monoclonal antibody. Immunoprecipitated HDAC7, MEF2D, and Twist2 were detected by western blotting.


A
IP:
anti-Flag
IgG
anti-Flag
IgG
anti-Flag
Input
IB: Flag
IB: HA
Flag-HDAC7 + + + + + + + +
HA-Twist2 - + + - - - + -
HA-Twist2CΔ - - - + + - - +

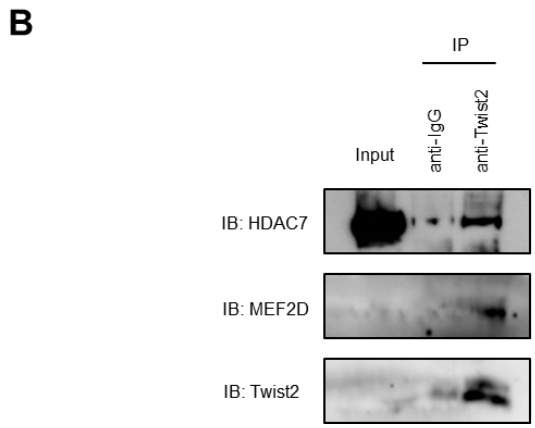

B
IP
Input
anti-IgG
anti-Twist2
IB: HDAC7
IB: MEF2D
IB: Twist2

Figure 24. Twist2, but not Twist2ΔC, represses *Nur77* promoter activity after TCR stimulation

16610D9 cells were transfected with indicated expression vectors. After 48 hr, transfected cells were further activated with non or PMA/IM. Relative luciferase activity to the results of PMA/IM stimulated mock transfected cells were summarized (error bars, ±SEM)

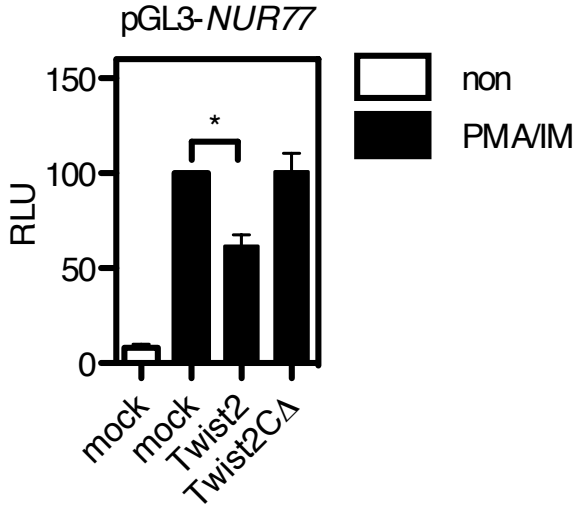
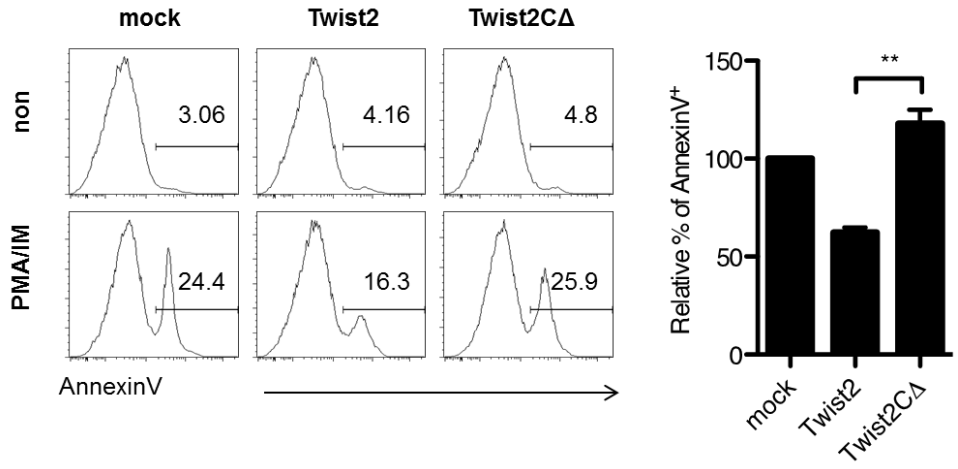

pGL3-*NUR77*
150
100
50
0
RLU
*
non
PMA/IM
mock
mock
Twist2
Twist2CΔ

Figure 25. Twist2, but not Twist2ΔC, represses AICD of thymocytes

16610D9 cells were transfected with either Twist2 or Twist2ΔC expression vector. At 48 hr, transfected cells were further activated with the PMA/IM treatment. Relative luciferase activities to the results of mock transfected cells were summarized (error bars, ±SEM).


mock
Twist2
Twist2CΔ
non
PMA/IM
3.06
4.16
4.8
24.4
16.3
25.9
AnnexinV
Relative % of AnnexinV+
150
100
50
0
**
mock
Twist2
Twist2CΔ

Twist2 retains HDAC7 in the nucleus after TCR stimulation

One of the remaining issues is how *Nur77* expression is differentially regulated depending on the TCR signal-strength. Previously suggested model, nuclear/cytoplasmic shuttling of HDAC7, is a plausible when this model adapted to the *Nur77* regulation in activated T cells. However, it was revealed that nuclear export of HDAC7 is occurred in positively-selected thymocytes as well as negatively-selected thymocytes. Thus, nuclear/cytoplasmic shuttling of HDAC7 could not distinguish the quantity of TCR signal-strength.

Since Twist2 is physically interacted with HDAC7, it is possible that enforced expression of Twist2 or up-regulated Twist2 in positively-selected thymocytes sequesters HDAC7 in the nucleus. To test this possibility, 16610D9 cells were transfected with Twist2 expression vector and stimulated with PMA/IM. The expression of HDAC7 in the nucleus was detected by western blotting assay after nuclear/cytoplasmic fractionation of cell extracts. Consistent with the previously reported data, the expression level of HDAC7 in the nucleus was decreased after PMA/IM stimulation (Figure 26) (Parra and others, 2007). However, when Twist2 was over-expressed, the expression level of HDAC7 in the nucleus was markedly increased (Figure 26). Increased HDAC7 protein level in the nucleus by Twist2 might be due to the result of increased HDAC7 expression in a transcriptional level. To rule out this possibility, HDAC7 expression in a transcript level was detected by semi-

qPCR with same samples used for detecting protein level of HDAC7 in the nucleus. Unlike the increased HDAC7 protein in the nucleus by Twist2, the expression of HDAC7 in a transcript level was not affected by Twist2 (Figure 27).

Interestingly, the protein expression level of HDAC7 in the nucleus was increased by Twist2 in unstimulated 16610D9 cells as well as activated 16610D9 cells (Figure 26). Though nuclear/cytoplasmic shuttling of HDAC7 is focused on the regulation of *Nur77* in the activated thymocytes, it was also possible that nuclear/cytoplasmic shuttling of HDAC7 presents in the resting thymocytes independent with TCR-signal strength because HDAC7 is located in both nucleus and cytoplasm. Thus, these results suggest that retaining HDAC7 in the nucleus by Twist2 is a consequence of physical interaction between Twist2 and HDAC7.

Twist2 retains HDAC7 in the nucleus of positively-selected thymocytes

Most DP thymocytes of TCR transgenic mice undergo positive selection and it was reported that HDAC7 is located in the cytoplasm of DP and SP cells from AND TCR-Tg and OT-I TCR-Tg mice (Kasler and others, 2011). To confirm these results suggesting HDAC7 localization at the cytoplasm in the positively-selected thymocytes, HDAC7 was detected in the nucleus of sorted

preDP and postDP thymocytes. Consistent with previous data, the expression of HDAC7 in the nucleus was decreased in postDP cells compared with preDP cells (Figure 28).

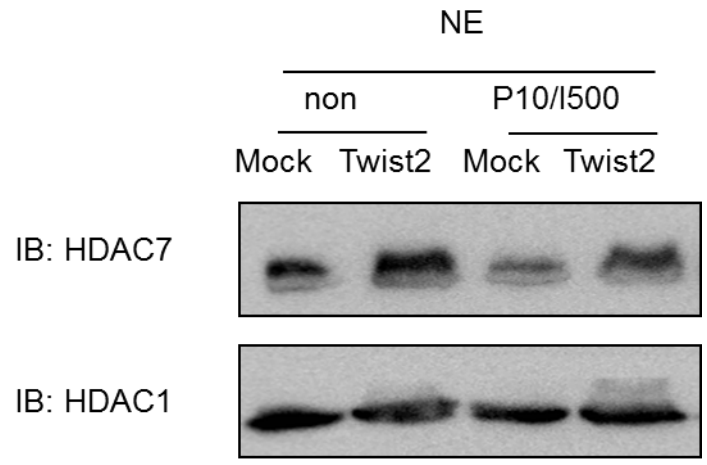
It is interesting that nuclear HDAC7 is still remained in postDP cells though the expression level of HDAC7 is lower than that in preDP cells (Figure 28). Since Twist2 retained HDAC7 in the nucleus of activated 16610D9 cells, Twist2 might sequester HDAC7 in the nucleus of positively-selected thymocytes. To substrate this possibility, HDAC7 expression was detected in the nuclear extracts of sorted positively-selected DP cells from OT-II TCR-Tg and OT-TCR x *Twist2*-Tg mice. Consistent with the results that the expression level of HDAC7 in the nucleus of activated 16610D9 cells was increased by Twist2, the amount of HDAC7 in the nucleus of positively-selected DP thymocytes was higher in OT-II TCR x *Twist2*-Tg mice than that in OT-II TCR-Tg mice (Figure 29).

Twist2 facilitates the physical interaction between MEF2D and HDAC7

Next issue is how Twist2 retains HDAC7 in the nucleus of positively-selected thymocytes. Considering the data showing that Twist2 interacted with both MEF2D and HDAC7, it is possible that Twist2 mediates the physical interaction between MEF2D and HDAC7.

Figure 26. Twist2 retains HDAC7 in the nucleus.

16610D9 cells were transfected with Twist2 expression vector. At 48 hr, transfected cells were further activated with PMA/IM. HDAC7 was detected in the nuclear extracts by western blotting.



* 16610D9 cells

Figure 27. HDAC7 expression is not affected by Twist2.

16610D9 cells were transfected with Twist2 expression vector. At 48 hr, transfected cells were further activated with PMA/IM and HDAC7 expression was detected by semi-qPCR. Twist2 expression was measured by using primer pairs detecting ORF region of Twist2.

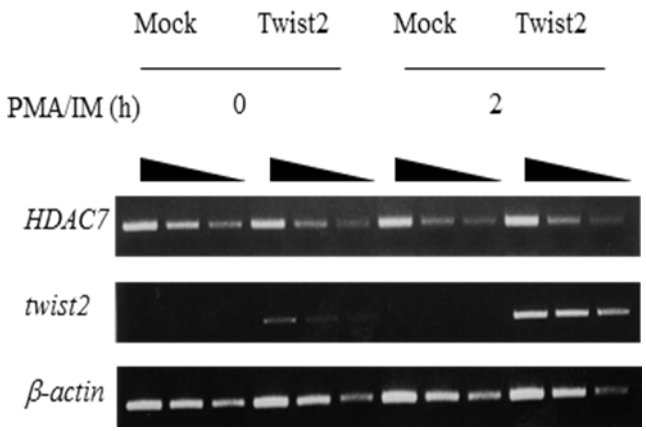

Mock
Twist2
Mock
Twist2
PMA/IM (h)
0
2
HDAC7
twist2
β-actin

Figure 28. Nuclear HDAC7 is decreased in the postDP cells compared with that in the preDP cells.

PreDP and postDP cells were sorted from C57BL/6 mice. HDAC7 expression was detected in the nuclear extracts of each populations by western blotting assay.

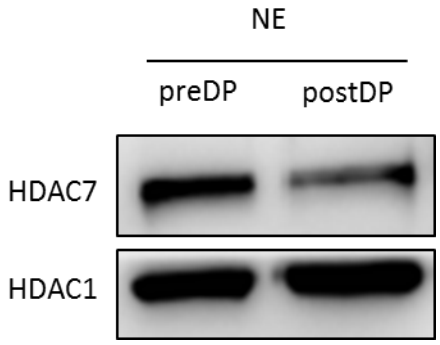
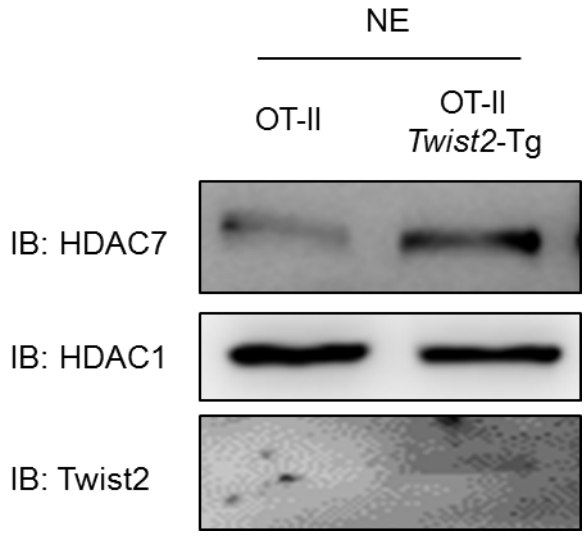

NE
preDP
postDP
HDAC7
HDAC1

Figure 29. Twist2 sequesters HDAC7 in the nucleus of positively-selected thymocytes.

DP thymocytes from indicated mice were sorted and HDAC7 expression was detected by western blotting assay in the nuclear extracts.


NE
OT-II
OT-II
Twist2-Tg
IB: HDAC7
IB: HDAC1
IB: Twist2

To detect the intensity of physical interaction between two proteins, BiFC assay is adapted. Because two VENUS subunits (VN and VC) are fused with each interested proteins using short linkage amino acids, the intensity of VENUS protein is positively correlated with physical interaction between two interested proteins. Additionally, by co-transfection with RFP-fused Twist2 expression vector (RFP-Twist2), the intensity of protein-protein interaction between MEF2D and HDAC7 can be detected depending on the expression level of Twist2 (Figure 30). To calculate the signal of VENUS fluorescence in a quantitative manner, VENUS fluorescence was detected by using FACS machine and calculated in four different regions (A to D) depending on the Twist2 expression levels. In mock transfected cells, the intensity of VENUS fluorescence was not correlated with RFP signal (Figure 30). However, fluorescence intensity of VENUS protein was increased by Twist2 depending on the expression levels of Twist2 (Figure 30).

Twist2 binding on the *Nur77* promoter is differentially regulated depending on the TCR signal-strength

Above data suggest that Twist2 recruits HDAC7 on the *Nur77* promoter. Besides, it seems that up-regulated Twist2 expression in positively selected thymocytes sequesters HDAC7 in the nucleus even though thymocytes are activated by TCR stimulation. Prior to clarify whether Twist2 recruits HDAC7 on the *Nur77* promoter, the binding pattern of Twist2 on the *Nur77* promoter

should be addressed. Though the expression of Twist2 was differentially regulated depending on the TCR signal-strength, Twist2 should bind to the MEF2 site of *Nur77* promoter to repress *Nur77* expression in low TCR signal-strength. Thus, it can be expected that Twist2 binding is higher in the weak stimulation than that in the strong stimulation. To test Twist2 binding, 16610D9 cells were stimulated with either P0.1/I200 (weak) or P10/I500 (strong) condition and Twist2 binding was detected by ChIP assay using anti-Twist2 antibody. Though Twist2 binding on the MEF2 site was increased in both weak and strong stimulation, the increased folds were significantly higher in the weak stimulation than that in the strong stimulation (Figure 31). Thus, these data suggest that Twist2 binding on the MEF2 site of *Nur77* promoter is differentially regulated depending on the TCR signal-strength.

Twist2 recruits HDAC7 on the MEF2 site of *Nur77* promoter

It has been well established the regulation mechanism of *Nur77* expression by HDAC7 in the resting and activated thymocytes. To repress *Nur77* expression, HDAC7 should bind to the *Nur77* promoter via the interaction with MEF2D because MEF2D binding domain-mutant HDAC7 failed to repress *Nur77* promoter activity and *Nur77* expression (Dequiedt and others, 2003). Though HDAC7/MEF2D interaction sufficiently explains the regulation mechanism if thymocyte statuses were simply dissected as resting and activated statuses, it could not demonstrate the differential expression

pattern of *Nur77* depending on the TCR signal-strength.

Since Twist2 binding was differentially regulated depending on the TCR signal-strength and Twist2 facilitated the interaction between MEF2D and HDAC7, these data strongly suggest that Twist2 regulates HDAC7 binding on the *Nur77* promoter depending on the TCR signal-strength. To validate this assumption, HDAC7 binding on the *Nur77* promoter was detected in the presence of Twist2 after TCR stimulation. Consistent with the previously reported results, the binding of HDAC7 on the *Nur77* promoter was declined after TCR stimulation (Figure 32) (Dequiedt and others, 2003). However, HDAC7 binding on the *Nur77* promoter was higher in the presence of Twist2 than that in the mock-transfected cells (Figure 32). Moreover, like increased nuclear localization of HDAC7 in both non-stimulated and stimulated 16610D9 cells transfected with Twist2 expression vector, HDAC7 binding was also increased in both non-stimulated and stimulated 16610D9 cells transfected with Twist2 expression vector (Figure 32). Consequently, TCR signaling-dependent Twist2 binding on the *Nur77* promoter recruits HDAC7 and thus regulates *Nur77* expression depending on the TCR signal-strength.

Twist2 binds to the HDAC7 site of *Nor-1* promoter and recruits HDAC7

Twist2 repressed *Nor-1* expression as well as *Nur77* expression in

activated thymocytes and the HDAC7 site on *Nor-1* promoter was pivotal for the repressive function of Twist2. As described previously, HDAC7 bound to the HDAC7 site in HH cutaneous T-cell lymphoma cell line. Since Twist2 repressed *Nor-1* promoter activity via the HDAC7 site, the binding of Twist2 on the HDAC7 site was investigated by ChIP assay. As expected, the binding of Twist2 on the HDAC7 site of *Nor-1* promoter was detected (Figure 33).

HDAC7 requires an anchor protein(s) to bind to the promoter because HDAC7 contains no DNA-binding domain (Lu and others, 2000; Wang and Yang, 2001). Unlike the *Nur77* promoter that MEF2D (and Twist2) serves as an anchor protein for HDAC7, there are no known proteins binding to the HDAC7 site, yet. Since Twist2 were detected as a binding protein on the HDAC7 site, Twist2 might serve as an anchor protein for the binding of HDAC7, there are no known proteins binding to the HDAC7 site, yet. Since Twist2 were detected as a binding protein on the HDAC7 site, Twist2 might serve as an anchor protein for the binding of HDAC7. To test this possibility, HDAC7 binding on the HDAC7 site was investigated by ChIP assay. HDAC7 binding on theHDAC7 site in 16610D9 cells were detected and this binding was decreased after TCR stimulation (Figure 34). However, Twist2 increased HDAC7 binding on the HDAC7 site in both non-stimulated and stimulated 16610D9 cells (Figure 34). Combining these data and the results showing Twist2 binding on the HDAC7 site, it is likely to address that Twist2 recruits HDAC7 on the HDAC7 site to repress the *Nor-1* promoter activity.

Figure 30. Twist2 facilitates the interaction between MEF2D and HDAC7.

A. VN-MEF2D and VC-HDAC7 vectors were co-transfected into 293T cells with either pDS-RED-C1 (RFP) or pDS-RED-C1-Twist2 (RFP-Twist2) expression vector. At 48 hr. fluorescence of each transfected samples were detected by FACS Canto II.

B. Relative fluorescence intensity of four regions (A to D) were summarized (error bars, ±SEM).

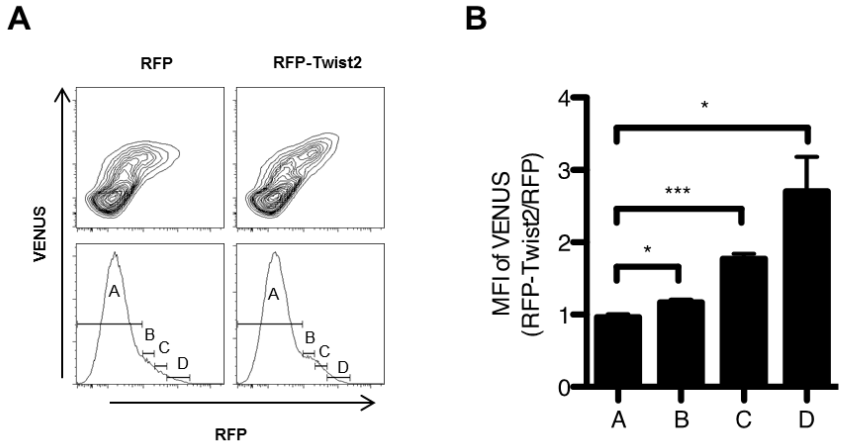

A
RFP
RFP-Twist2
VENUS
A
B
C
D
A
B
C
D
RFP
B
MFI of VENUS
(RFP-Twist2/RFP)
4
3
2
1
0
*
***
*
A
B
C
D

Figure 31. Twist2 binding on the *Nur77* promoter is differentially regulated depending on the TCR signal-strength.

16610D9 cells were stimulated with either P0.1/I200 or P10/I500 condition. Twist2 binding on the MEF site of *Nur77* promoter was detected by semi-qPCR and quantified using ImageJ software (error bars, ±SEM).

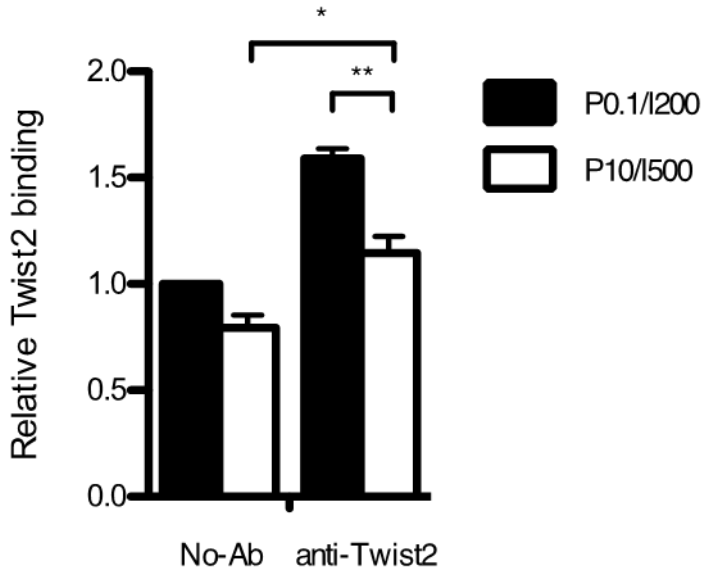
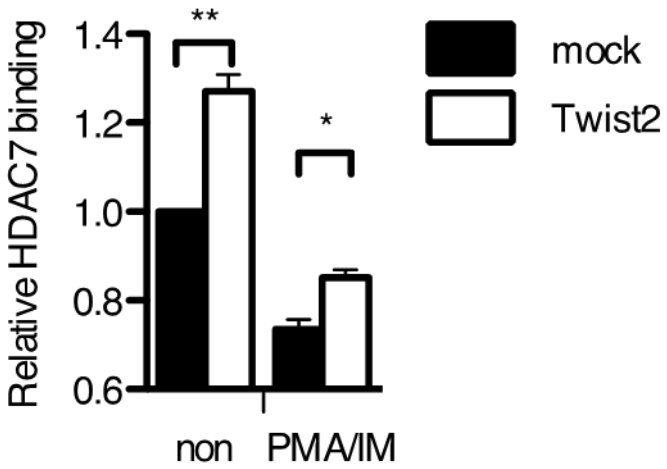

*
**
2.0
1.5
1.0
0.5
0.0
Relative Twist2 binding
P0.1/I200
P10/I500
No-Ab
anti-Twist2

Figure 32. Twist2 recruits HDAC7 binding on the MEF2 site of *Nur77* promoter

16610D9 cells were transfected with Twist2 expression vector. At 48 hr, transfected cells were stimulated with PMA. /IM. HDAC7 binding on the MEF site of *Nur77* promoter was detected by semi-qPCR and quantified using ImageJ software (error bars, ±SEM).


Relative HDAC7 binding
1.4
1.2
1.0
0.8
0.6
**
*
non
PMA/IM
mock
Twist2

Twist2 represses *Nur77* expression without HDAC7 enzymatic activity

It has been reported that Twist2 co-operates with HDACs to repress other gene expression (Gong and Li, 2002; Koh and others, 2009; Lee and others, 2003). In some cases, the treatment of TSA, a pan-HDAC inhibitor, completely abolished the suppressive function of Twist2 (Koh and others, 2009; Lee and others, 2003). However, in some cases, the TSA treatment only partially restored the suppressive function of Twist2. To determine whether HDAC7 enzymatic activity is necessary for the repressive function of Twist2 in the regulation of *Nur77* expression, *Nur77* expression was detected in the presence of Twist2. Prior to the PMA/IM stimulation, transfected cells were pre-treated with TSA for 30 min. Like previous reports, TSA treatment induced *Nur77* expression without TCR stimulation though induction folds were markedly lower than the *Nur77* expression level in PMA/IM-stimulated cells (Figure 35) (Dequiedt and others, 2005). Interestingly, Twist2 repressed *Nur77* induction after the treatments of both TSA and PMA/IM (Figure 35). In addition, Twist2 also repressed *Nur77* induction after PMA/IM plus TSA treatment (Figure 35). Together, these data suggest that Twist2 can repress *Nur77* expression without HDAC7 enzymatic activity.

Twist2 blocks p300 binding on the MEF2 site of *Nur77* promoter after TCR stimulation

As mentioned above, there are alternative ways of Twist2 repressive function without using HDAC enzymatic activity. Since Twist2 physically interacts with target gene promoter region and forms repressive function with other transcriptional repressors, Twist2 might compete out the binding chance of transcriptional activators in activated thymocytes. To speculate this possibility, the binding of p300, one of transcriptional activators inducing *Nur77* expression, on the *Nur77* promoter was detected in the presence of Twist2. Unlike HDAC7, which is released from the *Nur77* promoter after TCR stimulation, the binding of p300 was increased in the activated 16610D9 cells (Figure 36). However, the binding ability of p300 was decreased in the presence of Twist2 (Figure 36). Reduced p3000 binding by Twist2 might be due to the decreased expression of p300. To rule out this possibility, p300 expression in a transcriptional level was detected in *TCRα*$^{-/-}$ and *TCRα*$^{-/-}$ x *Twist2*-Tg thymocytes after PMA/IM treatment. The expression of p300 was comparable in the thymocytes between *TCRα*$^{-/-}$ and *TCRα*$^{-/-}$ x *Twist2*-Tg mice (Figure 37).

Taken together, these data suggest the Twist2/HDAC7 model in the regulation of *Nur77* expression during thymic selection processes (Figure 38). During positive selection, Twist2 expression is up-regulated and Twist2 binds

to the *Nur77* promoter. Though most of HDAC7 in the nucleus of resting thymocytes are exported after TCR signaling, a part of HDAC7 is sequestered by Twist2 on the *Nur77* promoter and forms a repressive complex. In contrast to the positive selection, Twist2 expression is low in the negatively-selected thymocytes and most HDAC7 is released from the *Nur77* promoter and empty *Nur77* promoter is replaced by transcriptional activators. Consequently, depending on the Twist2 expression level, *Nur77* expression is differentially regulated after TCR stimulation. In addition, Twist2 represses the expression of *Nor-1* as well as *Nur77*.

Figure 33. Twist2 binds to the HDAC7 site of *Nor-1* promoter.

ChIP assay was conducted to detect Twist2 binding on the HDAC7 site of *Nor-1* promoter in 16610D9 cells. The binding of Twist2 was detected by semi-qPCR.

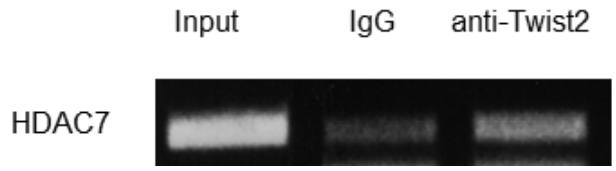

Input
IgG
anti-Twist2
HDAC7

Figure 34. Twist2 recruits HDAC7 on the HDAC7 site of *Nor-1* promoter.

16610D9 cells were transfected with Twist2 expression vector. At 48 hr, transfected cells were further activated with PMA/IM and ChIP assay was conducted. The binding of HDAC7 was detected by semi-qPCR.

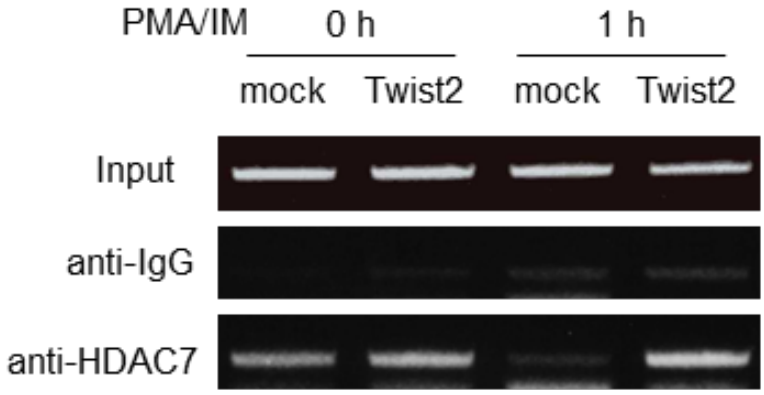

PMA/IM
0 h
1 h
mock
Twist2
mock
Twist2
Input
anti-IgG
anti-HDAC7

Figure 35. Twist2 represses *Nur77* expression without HDAC7 enzymatic activity.

16610D9 cells were transfected with Twist2 expression vector. At 48 hr, transfected cells were further activated with PMA/IM. TSA was treated 30 min prior to PMA/IM stimulation. The expression of *Nur77* was detected by qRT-PCR (error bars, ±SEM).

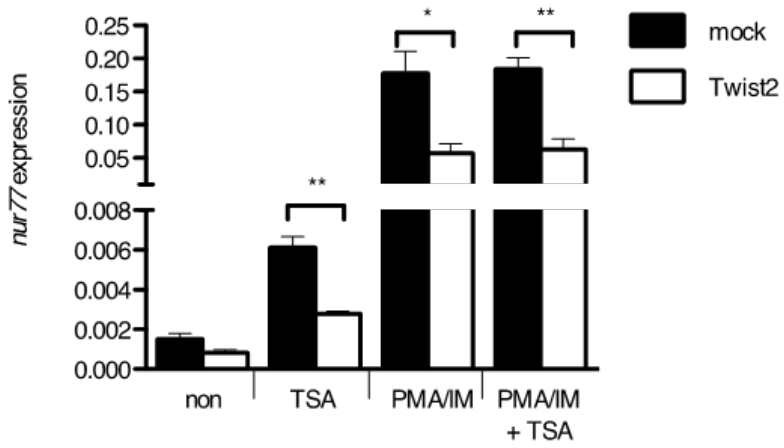

nur77 expression
0.25
0.20
0.15
0.10
0.05
0.008
0.006
0.004
0.002
0.000
*
**
**
mock
Twist2
non
TSA
PMA/IM
PMA/IM
+ TSA

Figure 36. Twist2 blocks p300 binding on the *Nur77* promoter after TCR stimulation

16610D9 cells were transfected with Twist2 expression vector. At 48 hr, transfected cells were further activated with PMA/IM. The binding of p300 was detected by semi-qPCR and quantified using ImageJ software (error bars, ±SEM).

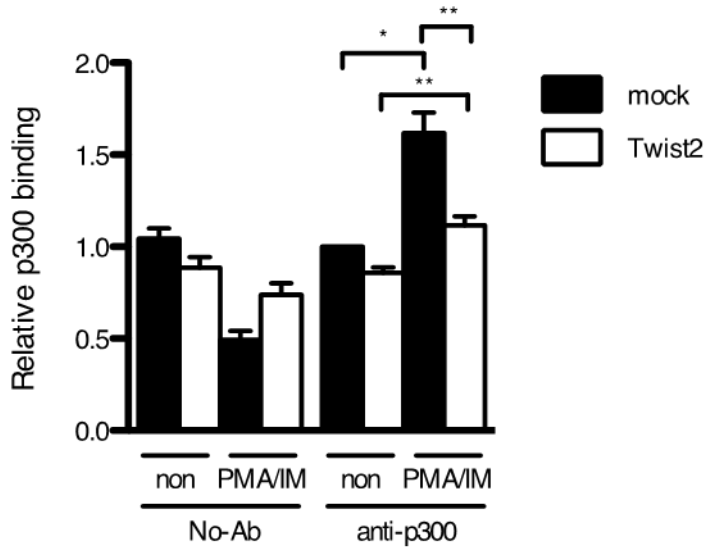

Relative p300 binding
2.0
1.5
1.0
0.5
0.0
*
**
**
mock
Twist2
non
PMA/IM
non
PMA/IM
No-Ab
anti-p300

Figure 37. The expression of p300 is not altered by Twist2.

Thymocytes from *TCRα*$^{-/-}$ and *TCRα*$^{-/-}$ x *Twist2*-Tg mice were activated with PMA/IM. The expression of p300 was detected by qRT-PCR (error bars, ±SEM).

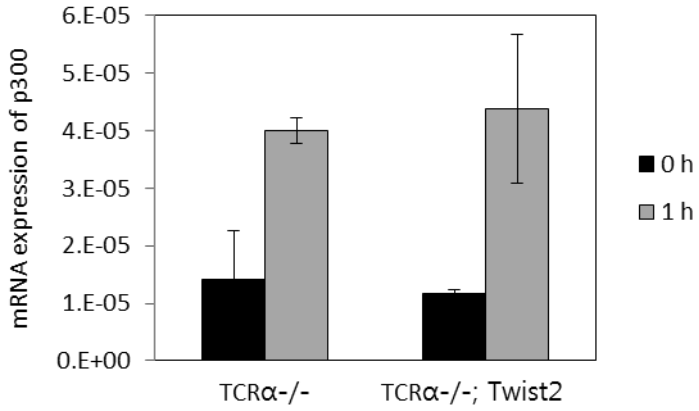
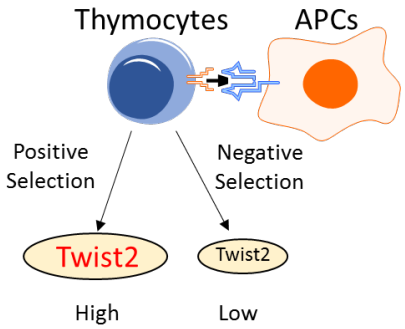
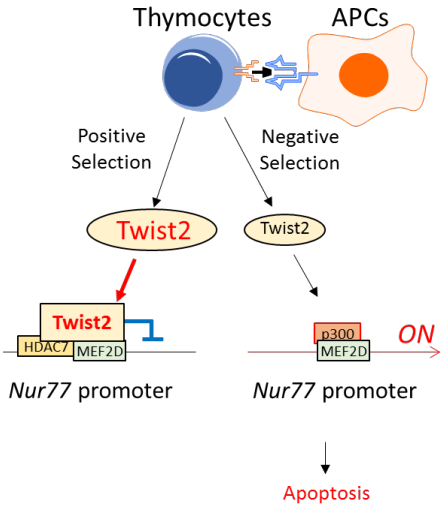
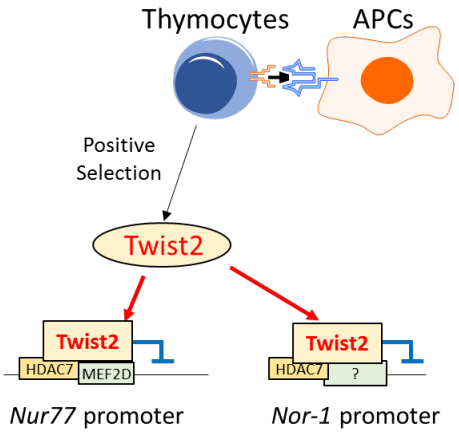

6.E-05
5.E-05
4.E-05
3.E-05
2.E-05
1.E-05
0.E+00
mRNA expression of p300
TCRα-/-
TCRα-/-; Twist2
0 h
1 h

Figure 38. The roles of Twist2 in thymic selection


Thymocytes
APCs
Positive Selection
Negative Selection
Twist2
Twist2
High
Low


Thymocytes
APCs
Positive Selection
Negative Selection
Twist2
Twist2
Twist2
HDAC7
MEF2D
p300
MEF2D
ON
Nur77 promoter
Nur77 promoter
Apoptosis


Thymocytes
APCs
Positive Selection
Twist2
Twist2
HDAC7
MEF2D
Twist2
HDAC7
?
Nur77 promoter
Nor-1 promoter

Real-time qRT-PCR	Forward primer	Reverse primer
twist2	5′-TGGACCAAGGCTCTCAGAACA-3′	5′-ACAGGAGTATGCGGGCAAGA-3′
nur77	5′-CCTGTTGCTAGAGTCTGCCTTC-3′	5′-CAATCCAATCACCAAAGCCACG-3′
nor-1	5′-AGACGCCGAAACCGATGT-3′	5′-CTCGGACAAGGGCATTCA-3′

ChIP locus	Forward primer	Reverse primer
nur77 promoter	5′-GCGGGCACGGATTTACAACACC-3′	5′-GGGTTCCATTGACGCAGGGAG-3′
nor-1 promoter	5′-CCCACCCTCCAAGAAGAAGTGAAGC-3′	5′-CCCACCCTCCAAGAAGAAGTGAAGC-3′
twist2 promoter	5′-GCCGAAAAAAGCGGAGACAAAACTG-3′	5′-GTCGCTCTCAAAGGCTCTGATTTCG-3′

Table 1. Primers for quantitative real-time PCR and ChIP assay

Discussion

T cells develop in the thymus and recognize pathogenic antigen in the periphery to induce immune response. In periphery, APCs present not only pathogenic antigens, but also present host-derived antigens. To make immune system balance between the diversity and the self-tolerance, thymocytes are educated in the thymus. Though it is not certain why immature thymocytes are more sensitive to TCR stimulation than mature T cells in the periphery, self-reactive thymocytes undergo apoptosis in the thymus. This process is called negative selection and builds a part of self-tolerance. Self-reactive thymocytes undergoing negative selection are eliminated by activation-induced cell death (AICD). If TCR signal-strength is over the threshold of the border between positive and negative selection, pro-apoptotic molecules such as Bim, Nur77, and Nor-1 are induced and triggers programmed cell death of thymocytes. However, how these pro-apoptotic molecules are suppressed in the positively-selected thymocytes are not clear, yet. In this report, the functions of Twist2 were studied in the thymocyte development, especially focused on the positive and negative selection.

Twist2 expression pattern during thymocyte development

Thymocyte developmental stages are well defined according to the changes of surface markers such as CD44, CD25, CD4, CD8, CD69, and TCRβ. Using the expression of CD4 and CD8 co-receptor, developmental

stages of thymocytes can be divided three stages, $CD4^-CD8^-$ (DN), $CD4^+CD8^+$ (DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ (SP), respectively, in an order. DN stages can be further dissected using CD44 and CD25 expression; $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4). DP stages can be further dissected using TCRβ and CD69; $TCR\beta^{lo}CD69^{lo}$ (preDP), $TCR\beta^{int/hi}CD69^{hi}$ (postDP). SP stages can be also divided using TCRβ and CD69; $TCR\beta^{hi}CD69^{hi}$ immature SP (iSP) and $TCR\beta^{hi}CD69^{lo}$ mature SP (mSP).

When Twist2 expression was investigated in these populations, Twist2 expression was high at DN stage and down-regulated at preDP stage. Twist2 expression was rebounded at preDP to postDP transition. During preDP to postDP transition, TCR α chain rearrangement is terminated and T cells undergo thymic selection processes depending on the TCR and pMHC interaction. *Twist2* expression seems to be regulated by TCR signaling because transcript level of *Twist2* was higher in sorted postDP cells than that in sorted preDP cells. Furthermore, Twist2 expression was increased when TCR unexperienced thymocytes (obtained from $TCR\alpha^{-/-}$ mice) were activated with PMA and ionomycin, which mimic distal TCR signaling. Another interesting finding in this experiment was that *Twist2* expression was differentially regulated depending on the TCR signal-strength. P0.1/I200 condition mimics positive selection, while P10/I500 condition mimics negative selection. Consequently, up-regulated *Twist2* expression after P0.1/I200 treatment and

down-regulated *Twist2* expression after P10/I500 treatment suggest the possibility that thymocytes sense the TCR signal-strength via the Twist2 expression level.

Twist2 regulates TCR sensitivity of thymocytes

Twist2 expression is high in the positively-selected thymocytes, while down-regulated in the negatively-selected thymocytes. It is possible that Twist2 might assist the survival of thymocytes and/or repress the cell death program. In order to survive positively-selected thymocytes, pro-survival molecules such as Bcl-2, Bcl-X_L, and Mcl-1 are required. However, the expression of these pro-survival molecules was not changed in the presence of Twist2. Thus, it seems that Twist2 inhibits cell death pathway during thymic selection process rather than regulating gene expressions associated thymocyte survival. It was also reported that Twist2 acts as a potential oncogene that inhibits MYC-induced apoptosis (Maestro and others, 1999).

Based on the Twist2 expression pattern depending on the TCR signal-strength, it is likely assumed that Twist2 inhibits cell death program in the positively-selected thymocytes, while down-regulation of Twist2 in the negatively-selected thymocytes triggers cell death program. From these assumptions, following results are expected; activated thymocytes by TCR-stimulation undergo apoptosis in the absence of Twist2 and/or survive in the presence of Twist2. Increased TCR-sensitivity in the absence of Twist2 is

supported by following results. First, the apoptotic cells in both $TCR\beta^{int/hi}CD69^{hi}$ and $TCR\beta^{hi}CD69^{lo}$ population were increased in *Twist2* cKO mice. Since these two populations are receiving or received TCR stimulation, it seems that apoptosis in TCR experienced thymocytes are increased in *Twist2* cKO mice. Second, DP cell viability after antigen-stimulation was decreased in *Twist2* cKO mice. DP cells are much more sensitive to TCR stimulation than any other population in the thymus (Davey and others, 1998). Thus, when whole thymocytes are stimulated with cognate antigen, DP cells are particularly eliminated by TCR stimulation and the percentage of DP population is decreased (Staton and others, 2011). Thus, the decreased the DP cell viability in *Twist2* cKO mice also supports that TCR-sensitivity of thymocytes is increased in the absence of Twist2. Third, PMA/IM-induced AICD was increased in the thymocytes of *Twist2* cKO mice. TCR signaling is very complicated and many components are associated in the signal pathways from TCR to the induction of pro-apoptotic genes. Fortunately, the treatment of PMA and ionomycin mimics the distal TCR signaling, which can be simply divided as PKC and Ca^{2+} pathway, and high concentration of PMA/IM causes AICD of thymocytes. Thus, the PMA/IM treatment is able to preclude the effect of proximal signaling components. Collectively, these data suggest that TCR-sensitivity of thymocytes is increased in the absence of Twist2.

Another expected result is the repression of thymocyte AICD in the presence of Twist2. If down-regulation of *Twist2* expression in the negatively-

selected thymocytes is essential for the initiating cell death program, enforced expression of *Twist2* might block the apoptosis of negatively-selected thymocytes. There are many mice models to investigate the negative selection of thymocytes. One of them is RIP-mOVA-Tg mice system. RIP-mOVA-Tg mice express ovalbumin in the pancreas, kidney, and thymic medulla. Thymocytes from OT-II TCR-Tg and DO11.10 TCR-Tg mice can recognize OVAp presented on APCs and undergo apoptosis by negative selection. As expected, negative selection in this mice system was impaired in *Twist2*-Tg mice. Reduction of mature OT-II TCR^+ ($CD24^{lo}V\alpha2^+$) thymocytes was disappeared in *Twist2*-Tg mice. One of the interesting findings is that Twist2 represses Nur77 expression under RIP-mOVA-Tg background. It was reported that negative selection of OT-II TCR x RIP-mOVA-Tg mice model is attenuated in the *Nur77*-null background (Fassett and others, 2012). Thus, these data suggest that inhibition of negative selection in Twist2-Tg mice is due to the repression of Nur77 expression.

Twist2 represses the expression of *Nur77*, but not *Bim*

Both Nur77 and Bim are well known pro-apoptotic molecules inducing AICD of thymocytes after TCR stimulation. However, there are controversies in the roles of Nur77 and Bim in negative selection. One of famous negative selection mice model is H-Y TCR-Tg mice system. Thymocytes from male mice of H-Y TCR-Tg mice recognize a y-chromosomal peptide in the context of $H\text{-}2D^b$ and undergo apoptosis at DP stage (Kisielow and others, 1988),

while thymocytes from female mice of H-Y TCR-Tg are positively selected into the CD8 lineage cells (Teh and others, 1988). It was reported that negative selection in H-Y TCR-Tg male mice was impaired in transgenic mice expressing dominant-negative form of Nur77 (Zhou and others, 1996) and Bim-deficient mice (Bouillet and others, 2002). However, unlike the increase peripheral H-Y TCR^+ CD8 T cells, escaping from negative selection, in DN-*Nur77*-Tg mice, peripheral H-Y TCR^+ CD8 T cells were not detected in *Bim*$^{-/-}$ mice. Instead, these cells were accumulated in the thymus showing a phenotype, $CD4^{lo}CD8^{lo}$ (Kovalovsky and others, 2010). In this paper, the authors sorted $CD4^{lo}CD8^{lo}$ population and noticed that *Nur77* expression was increased in this population. These data suggest that Nur77 and Bim are both required for the elimination of autoreactive thymocytes in early developmental stages including DP stage, while Nur77, but not Bim, is required for the elimination of autoreactive thymocytes in later stage like $CD4^{lo}CD8^{lo}$.

According to above data, it seems that Nur77 and Bim plays independently due to the thymocyte developmental stages, Bim in early stage and Nur77 in late stage. However, recently, it was reports that *Bim* expression is down-regulated in the thymocytes of *Nur77*$^{-/-}$ mice (Kasler and others, 2012). In addition, synergistic effect in the positive and negative selection of *Bim* and *Nur77* double knockout mice was reported (Hu and Baldwin, 2015). In this report, *Nur77*$^{-/-}$ mice showed mild phenotypes in positive and negative selection. However, *Nur77* and *Bim* double knockout mice displayed abnormal

negative selection and enhanced positive selection. Though it is uncertain how Nur77 and Bim co-operate in the regulation of thymic selection processes, precise regulation of these two molecules is crucial in the thymocyte development.

In *Twist2* cKO mice, the frequency of TCRβ^{hi}CD69^{lo} population was decreased compared with control mice, whereas the frequency of TCRβ$^{int/hi}$CD69^{hi} population was comparable between *Twist2* cKO and control mice. Since *Twist2*-deficiency increased TCR-sensitivity of TCR-unexperienced DP thymocytes, this phenotypical discrepancy is quite interesting. In TCRβ$^{int/hi}$CD69^{hi} population, postDP and iSP cells are included. These cells are located in the thymic cortex. TCRβ^{hi}CD69^{lo} population is confused with HSA^{hi} immature SP cells and HSA^{lo} mature SP cells. Location of HSA^{hi} immature SP cells and HSA^{lo} mature SP cells are different. HSA^{hi} immature SP cells are transition stage from cortex to medulla, while HSA^{lo} cells are located in the medulla and ready to egress to the periphery. Considering the results that T cells escaping negative selection in DN-Nur77-Tg mice, but not *Bim*$^{-/-}$ mice, were able to egress to the periphery, negative selection of the thymocytes in the late stage of thymocyte development, decreased frequency of TCRβ^{hi}CD69^{lo} population in *Twist2* cKO mice might reflect the consequences that Twist2 repressed the expression of *Nur77*, but not *Bim*.

Twist2 simultaneously regulates the expression of NR4A receptor family members, *Nur77* and *Nor-1*

Nur77 expression was induced by TCR stimulation and *Nur77*-Tg mice exhibited massive cell death of thymocytes. However, *Nur77*-null mice displayed normal negative selection. Later, it was revealed that these discrepancies were due to the functional redundancy between Nur77 and Nor-1. Transgenic mice expressing dominant-negative form of Nur77 protein, which blocks the functions of both Nur77 and Nor-1, showed abnormal negative selection. The phenotype of *Nur77* and *Nor-1* double knockout mice was also reported (Sekiya and others, 2013). In this report, authors showed that Treg generation was more severely impaired in *Nur77* and *Nor-1* double knockout mice than that in each single knockout mice. Though they did not address the synergistic effect in negative selection of *Nur77* and *Nor-1* double knockout mice, this report supports the idea that Nur77 and Nor-1 have a functional redundancy in the thymocyte development. Since *Nor-1*-Tg mice also displayed massive cell death of thymocytes like the same phenotype in *Nur77*-Tg mice, simultaneous regulation of *Nur77* and *Nor-1* expression is required for the suppression of thymocyte AICD.

Nuclear exportation of HDAC7 is critical for the induction of *Nur77* expression because HDAC7 mutant (HDACΔP) at the phosphorylation sites required for the nuclear exportation failed to induction of *Nur77* expression after TCR stimulation (Kasler and others, 2012). In this report, authors showed

that HDAC7ΔP repressed the expression of Nor-1 as well as Nur77 though they did not address whether HDAC7 directly regulates the expression of Nor-1. Instead, they showed that TCR signaling was attenuated in the thymocytes of HDAC7ΔP mice. Unlike HDAC7ΔP-Tg mice, TCR signaling was normal in *Twist2*-Tg mice. Since Twist2 repressed both *Nur77* and *Nor-1* expression without altering TCR signaling, Twist2 seems to be directly regulating both *Nur77* and *Nor-1* expression. This idea is supported by several data. First, Twist2 can bind to the promoters of *Nur77* and *Nor-1* via the MEF2 site and the HDAC7 site, respectively. Second, the promoter activities of mutant constructs at these elements were not regulated by Twist2. Third, Twist2 recruited HDAC7 on the both *Nur77* and *Nor-1* promoter. In addition, Twist2 blocks the p300, a transcriptional activator of *Nur77* induction, binding on the *Nur77* promoter. Collectively, these data suggest that Twist2 directly regulates the expression of *Nur77* and *Nor-1* at the same time.

Twist2 co-operates with HDAC7 to regulate *Nur77* expression

Nuclear HDAC7 is phosphorylated after TCR stimulation and exports to the cytoplasm. It was reported that HDAC7 was localized in the cytoplasm of positively-selected thymocytes, which expresses low level of *Nur77* expression (Kasler and others, 2011). The result presented in Figure 28, however, depicts that HDAC7 was partially remained in the nucleus of postDP cells, which are activated by TCR stimulation, though the amount of HDAC7 was decreased compared with that in the preDP cells. Since Twist2 expression

was higher in the postDP cells than preDP cells and Twist2 physically interacted with HDAC7, it was possible that Twist2 sequesters HDAC7 in the nucleus in the positively-selected thymocytes. To directly investigate the expression level of HDAC7 in the nucleus of positively-selected thymocytes, HDAC7 expression was detected in the DP thymocytes of TCR-Tg mice because most DP thymocytes are undergoing positive selection. As expected, the amount of nuclear HDAC7 was higher in the DP thymocytes of *Twist2* x DO-Tg mice than that of DO-Tg mice. Furthermore, the binding of HDAC7 on the *Nur77* promoter in TCR-Tg thymocytes was higher in Twist2-Tg mice than that in NLC mice. Taken together, these data suggest that Twist2 sequesters HDAC7 on the *Nur77* promoter in the positively-selected thymocytes.

Another interesting finding is that Twist2 repressed the expression of *Nur77* promoter without HDAC enzymatic activity. It is controversial whether Twist2 requires HDAC enzymatic activity to repress other gene expression (Gong and Li, 2002; Koh and others, 2009; Lee and others, 2003). In *Nur77* regulation, however, it seems that Twist2 did not require HDAC enzymatic activity. Instead, physical interaction between Twist2 and HDAC7 has a responsibility on the regulation of *Nur77* expression because Twist2ΔC, a deletion mutant of HDAC7 binding domain, failed to repress the *Nur77* promoter activity and apoptosis of thymocytes after TCR stimulation. Additionally, HDAC7 binding on the *Nur77* was significantly remained on the

Nur77 promoter in the presence of Twist2 even after TCR stimulation. Thus, these data suggest that Twist2 facilitates the formation of repressive complex on the *Nur77* promoter during positive selection. The result that Twist2 blocks the binding of a transcriptional activator, p300, further supports this Twist2/HDAC7 model in the regulation of *Nur77* expression.

"Twist2/HDAC7 model" in the regulation of thymocyte development

Twist2/HDAC7 model explains many unresolved questions in the regulation mechanism of *Nur77* expression. First of all, Twist2/HDAC7 model can address the TCR signal-strength-dependent *Nur77* expression, which could not be explained by nuclear/cytoplasmic shuttling model of HDAC7. Second, Twist2/HDAC7 model can explain the Ca^{2+} pathway-dominant *Nur77* induction. Unlike the nuclear export of HDAC7 is mediated by PMA treatment, which activates PKC pathway, Twist2 expression is higher in low concentration of ionomycin (a Ca^{2+} ionophore) than that in high concentration.

The regulation mechanism of cell death pathway in negatively-selected thymocytes by Twist2 is clearly addressed. However, further studies are needed to define the roles of Twist2 in positively-selected thymocytes. *Twist2* cKO mice exhibited decreased thymocyte cellularity in polyclonal TCR condition, but not in TCR-Tg condition (Figure 5 and Figure 9). These discrepancies might be due to the earlier selection process in TCR-Tg mice

than polyclonal TCR condition. Most TCR-Tg construct uses *Lck*-promoter, including OT-II TCR-Tg and DO-Tg mice. *Lck*-promoter is activated from DN2 stage. Non-physiological expression of TCR transgenes are interacted with pMHC at DN stage and transduces TCR signal aberrantly though most TCR-Tg thymocytes undergo positive selection. It was reported that *Hdac7* cKO mice displayed shorten-life span of DP thymocytes (Kasler and others, 2011). Thus, though one of the reasons of the reduction of thymic cellularity in *Twist2* cKO mice under polyclonal TCR condition is increased apoptosis of post-selected thymocytes, another role of Twist2 might be added the *Twist2* cKO phenotype like shorten-life span of DP thymocytes in *Hdac7* cKO mice.

Another interesting finding is related with T_{reg} generation in the thymus. Recently, the roles of NR4A receptor family members are emerged in T_{reg} generation and the induction of Foxp3 expression in T_{reg} cells (Sekiya and others, 2013). The authors addressed that the generation of Treg cells were completely disappeared in triple-knockout, *Nur77*, *Nurr1*, and *Nor-1*, mice. Since TCR signal is associated with T_{reg} generation at least in a part, Twist2 might play in the generation of T_{reg} cells by regulating the expression of NR4A receptor family members. Further studies will be required to delineate the roles of Twist2 in T_{reg} generation.

Twist2 might also play in early T cell development because Twist2 expression was high at DN cells. *Twist2* was specifically deleted in the thymocytes using *Cd4*-Cre-Tg mice and *Twist2* deletion was detected at preDP

stage, but not DN stage. For this reason, knockout effect of *Twist2* in the DN stage can be precluded in the interpretation of the results obtained from *Twist2* cKO mice. However, the presence of Twist2 roles in early T cell developments are expected because there are lots of papers the function of other E-proteins such as E2A and Id3 were reported (Engel and others, 2001; Engel and Murre, 2004; Lauritsen and others, 2009). Thus, if Twist2-floxed mice were crossed with *Lck*-Cre mice, the roles of Twist2 in the early thymocyte developmental stage will be fully understood.

Here, I suggest the "Twist2/HDAC7 model" to speculate the regulation of *Nur77* expression during thymic selection processes (Figure 38). In positive selection, Twist2 is highly expressed and binds to the *Nur77* promoter. Though most of HDAC7 in the nucleus of resting thymocytes are exported after TCR signaling, a part of HDAC7 is sequestered by Twist2 on the *Nur77* promoter and forms a repressive complex. In contrast to the positive selection, Twist2 expression is low in the negatively-selected thymocytes and most HDAC7 is released from the *Nur77* promoter and empty *Nur77* promoter is replaced by transcriptional activators. Consequently, *Nur77* expression is differentially regulated depending on the Twist2 expression level with a co-operation with HDAC7.

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국문 초록

전사조절 인자인 Twist2에 의한 흉선 T세포의 부정적 선별과정에 관한 연구

오 상욱

자가항원에 반응성을 보이는 T세포는 흉선에서 T세포가 발생하는 동안 부정적 선별과정에 의하여 제거된다. 자가항원에 강하게 반응할 수 있는 T세포 수용체(T-cell receptor; TCR)를 가진 흉선 T세포는 Nur77과 Nor-1을 발현하고 자가세포사멸(apoptosis)된다. 이 두 유전자의 발현과 그 중요성에 대해서는 많은 연구가 수행되었지만, 기능적 유사성을 보이는 이 두 유전자의 발현을 동시에 조절하는 방법은 아직 알려지지 않았다. 또한 *Nur77*의 발현을 억제한다고 알려진 HDAC7이 없어도 *Nur77*의 발현이 증가되지 않는다는 사실이 최근에 보고되면서, *Nur77*과 *Nor-1*을 조절할 새로운 전사인자에 대한 연구가 필요하다. Twist2는 bHLH 군에 속하는 전사인자로 *Nur77*과 *Nor-1*의 발현을 억제하여 흉선 T세포의 부정적 선별과정을 억제한다는 사실을 발견하였다. 또한 Twist2가 *Nur77* 프로모터 결합하여 전사억제인자인 HDAC7의 결합을 촉진하고 전사촉진인자인 p300의 결합을 방해한다는 사실을 확인하였다. *Twist2*의 발현은 TCR 신호의 강도에 따라 다르게 조절되며, 특히 긍정적 선별과정을 유도하는 TCR 신호에 의해서는 높게 발현되고, 반면 부정적 선별과정을 유도하는 TCR 신호에 의해서는 발현이 억제되었다. 이를 통해,

TCR 신호의 강도에 따라 *Twist2*의 발현이 조절되고, Twist2가 *Nur77*과 *Nor-1*의 발현을 동시에 통제하여 흉선의 부정적 선별과정을 조절하는 역할을 수행한다는 사실을 규명할 수 있었다.

주요어: Twist2, Nur77, Nor-1, 흉선 T세포,
부정적 선별과정, 아포토시스, HDAC7

학번: 2007-20349